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RNAi and expression of retrotransposons MuERV-L and IAP in preimplantation mouse embryos

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Abstract

Both murine endogenous retrovirus-L (MuERV-L) and intracisternal A particle (IAP), two autonomous long terminal repeat (LTR) retrotransposons, are activated during genome activation in the preimplantation mouse embryo, and both sense and antisense transcripts are detected in 2-cell and 8-cell stage embryos. Because RNA interference (RNAi) functions in the preimplantation mouse embryo, we analyzed the relationship between RNAi and MuERV-L and IAP expression by inhibiting RNAi and measuring relative changes of the levels of these transcripts. We inhibited the initial step in the RNAi pathway by injecting 1-cell embryos with mDicer siRNA or long mDicer dsRNA and analyzed MuERV-L and IAP expression at the 8-cell stage. This approach resulted in the targeted destruction of mDicer mRNA, but not Hdac1 mRNA, inhibited the RNAi pathway, and resulted in a 50% increase in IAP and MuERV-L transcript abundance. These results suggest that RNAi constrains expression of repetitive parasitic sequences in preimplantation embryos, and thereby contributes to preserving genomic integrity at a stage of development when the organism consists of only a few cells.

Keywords: RNAi; Retrotransposon; Dicer; Preimplantation embryo; Genome activation

Introduction

RNA interference, known as RNAi, is a conserved eukaryotic mechanism in which dsRNA induces sequence-specific degradation of homologous mRNAs. RNAi is initiated by cleavage of dsRNA into 21–23 nucleotide short interfering RNAs (siRNA) by the RNase III enzyme Dicer (Bernstein et al., 2001), and the siRNAs produced by Dicer direct the recognition and degradation of the homologous mRNA (Martinez et al., 2002). RNAi operates in mouse oocytes and preimplantation embryos (Svoboda et al., 2000, 2001; Wianny and Zernicka-Goetz, 2000) but its biological role during preimplantation development is not known.

RNAi is associated with the suppression of transposable elements in several species. For example, transposable elements are mobilized in some RNAi-resistant mutants in *Caenorhabditis elegans* (Ketting et al., 1999) and transposon "taming" in *Drosophila* is homology-dependent and mediated by RNA (Jensen et al., 1999). Of particular interest is that siRNAs derived from retrotransposons are found in *Trypanosoma* (Djikeng et al., 2001) and *Drosophila* (Caudy and Hannon, 2002). Members of the Argonaute family are linked to the RNAi pathway, and *Trypanosoma* deficient in AGO1 have reduced amounts of retrotransposon-derived siRNAs and increased abundance of retrotransposon transcripts (Shi et al., 2003). In addition, RNAi is involved in transposon silencing in the germline of *C. elegans* (Sijen and Plasterk, 2003). The nexus between retrotransposon silencing and dsRNA-induced RNA interference, however, is not complete. The absence of mobilization of transposable elements in strong RNAi mutants such as *rde-1* and *rde-4* in *C. elegans* suggests that there might be differences between transposable element silencing and the RNAi pathway (Tabara et al., 1999), that is, transposable elements may be recognized and directed to the RNAi pathway differently

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than dsRNA fed or injected into animals (Tabara et al., 2002). Nevertheless, the aforementioned results strongly suggest that a conserved RNAi-related mechanism generating siRNAs operates on transposable elements in many eukaryotes. In addition, a well-known mammalian line of defense against transposable elements is repression of transcription associated with DNA methylation (Walsh et al., 1998).

In general, genomes of higher eukaryotes are replete with repetitive sequences derived from mobile elements; approximately 40% of the human and mouse genomes are occupied by four major classes of interspersed repetitive elements that are related to transposable elements (Lander et al., 2001; Waterston et al., 2002). Most of these sequences come from retroelements, transposable elements that transpose through a "copy and paste" mechanism(s) utilizing an RNA intermediate. Employing a "copy and paste" mechanism, retroelements can rapidly increase their copy number in the genome (in contrast to DNA transposons, which transpose through a "cut and paste" mechanism). Nevertheless, although retroelements are capable of being expressed efficiently the vast majority bear various mutations, truncations, and deletions (Sassaman et al., 1997; Turner et al., 1992).

We report here our study of the role of RNAi in constraining the expression of repetitive elements in the preimplantation mouse embryo. Microinjection of either mDicer siRNA or dsRNA into 1-cell embryos results in the targeted destruction of mDicer mRNA by the 8-cell stage and inhibits the RNAi pathway in preimplantation embryos. This inhibition of RNAi correlates with an increase in the mRNA levels of intracisternal A particle (IAP) and murine endogenous retrovirus-L (MuERV-L), for which antisense transcripts are detected from the 2-cell stage and onward. Therefore, one function of RNAi in mammals may be to constrain the expression of repetitive parasitic mRNA sequences during preimplantation development, which could limit transposition and preserve genomic integrity at a stage of development when an organism consists of only a few cells.

Materials and methods

Oocyte and embryo collection, culture, microinjection, and image analysis

Oocyte and embryo collection, culture, and microinjection were performed as previously described (Doherty et al., 2000; Svoboda et al., 2000). Embryos were obtained from 6-week old CF1 female mice (Harlan) mated to B6D2F1/J males (Jackson Laboratories, ME). Females were superovulated by an intraperitoneal (i.p.) injection of PMSG (5 IU) followed by an i.p. injection of human chorionic gonadotropin (hCG; 5 IU) 48 h later. Embryos were cultured in KSOM medium (Erbach et al., 1994) in an atmosphere of 5% CO_2/5% O_2/90% N_2. One-cell embryos were microinjected in bicarbonate-free Whitten's medium (Whitten, 1971) containing 25 mM HEPES, pH 7.2, and 0.01% PVA. Images were captured using a Nikon Eclipse TE2000 inverted microscope and EGFP fluorescence intensity was quantified using MetaMorph software (Universal Imaging Corporation, Downingtown, PA).

Homozygous transgenic male mice containing an Oct4 promoter-driven EGFP transgene were used to generate the 1-cell embryos that were microinjected with EGFP dsRNA, mDicer siRNA, or mDicer dsRNA in the experiments described in Fig. 4. The male transgenic mice were a generous gift of John McLaughlin and Hans Schöler (Boiani et al., 2002). EGFP and mDicer dsRNA were prepared as previously described (Stein et al., 2003a); the mDicer dsRNA corresponded to nucleotides 33–1521 (GenBank accession number AF43085).

RNA isolation from microinjected samples and RT-PCR

RNA was isolated from oocytes and early embryos as described previously (Temeles et al., 1994) or using magnetic beads (Dynal Biotech) according to the manufacturer's microscale protocol. Typically, RNA from 20 to 25 oocytes was isolated. Before RNA isolation, 0.125 pg rabbit β-globin mRNA per oocyte was added to the samples as an external standard. rRNA (20 μg) was then added to each sample as carrier. The RNA, which was precipitated with ethanol and pelleted by centrifugation, was re-suspended in 5% RNasin (Promega) in DEPC-treated water. For reverse transcription, total RNA or poly(A) RNA (eluted from the magnetic beads) corresponding to at least 18 oocytes or embryos (typically 20–25) was reverse-transcribed with Superscript II (Life Technologies) using the manufacturer's protocol. Following reverse transcription, two oocyte or embryo equivalents were used as a template for each PCR reaction. PCR products were radiolabeled with [α^{32}P] dCTP (50 μCi/ml, Amersham, 3000 Ci/mmol).

For each set of gene-specific primers, the linear region of semi-log plots of the amount of PCR product as a function of cycle number was determined and a cycle number for each primer pair was selected that was in this linear range. This method permits the comparison of relative changes in the abundance of a particular transcript (Temeles et al., 1994). After PCR, the products were subjected to electrophoresis in a nondenaturing 6% polyacrylamide gel. The gel was dried under vacuum for 1 h at 80°C, exposed in a phosphorimager cassette for 4 to 24 h, and the signal was quantified using a Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics).

All primers used in this study are shown in Table 1. Except for the globin primers, conditions for every other PCR reaction included denaturation at 94°C for 30 s followed by 30 s of annealing and then a 1-min extension at 72°C. Conditions for globin PCR were: denaturation at

Table 1
Primers used for RT-PCR

Name	Sequence	T_m (°C)	Cycle no.
MuERV-A.F	CCATCCCTGTCATTGCTCA	For reverse transcription	
MuERV-A.R	CCTTTTCCACCCCTTGATT	For reverse transcription	
MuERV-B.F	TGGTGGTCGAGATGGAGGTTA	55	30
MuERV-B.R	CCGTGAATGGTGGTTTTAGCA	55	
IAP-A.F	GACCCACCTGACTGGACTT	For reverse transcription	
IAP-A.R	AACGCTGCTCTCTCTTTAT	For reverse transcription	
IAP-B.F	GCACCCTCAAAGCCTATCTTAT	55	28
IAP-B.R	TCCCTTGGTCAGTCTGGATTT	55	
HDAC1.F	ACCAACCAGAACACTAACGAG	59	32
HDAC1.R	TTGAGGGAGAGTGAGGAAC	59	
globin.F	GCAGCCACGGTGGCGAGTAT	60	22
globin.R	GTGGGACAGGAGCTTGAAAT	60	
mDicer.F	TGGCACCAGCAAGAGACTCA	55	36
mDicer.R	CTGGGAGATGCGATTTTGGA	55	

95°C for 10 s, followed by annealing and extension at 62°C for 15 s. PCR primers for amplification of transposable elements were generated from the most conserved sequences among cDNAs found in early embryo cDNA libraries in Genbank. Therefore, these primers allowed for the simultaneous evaluation of expression of transposable elements at many loci. MuERV-L and IAP primers amplify sequences at the 3′ end of the respective elements (Fig. 2A).

For the analysis of temporal pattern of expression of mDicer, IAP, and MuERV-L, total RNA from approximately 100 oocytes, eggs or preimplantation embryos was isolated and reverse-transcribed (Temeles et al., 1994). cDNA corresponding to five embryos (five embryo-equivalents) was used in each PCR reaction.

For the RT-PCR analysis of sense and antisense strand expression, total RNA was isolated from 250 embryos. Fifty embryo-equivalents were used for strand-specific reverse transcription and five embryo equivalents were used for 36 cycles of PCR (nonlinear conditions).

siRNA and dsRNA used for microinjection

siRNA duplexes against mDicer and luciferase (Luciferase GL2) were purchased from Dharmacon Research Inc. (Lafayette, CO). Two different siRNAs were used to target mDicer: 5′-tcaaccagccactgctgga-3′ and 5′-catgaaggccgcctttcat-3′, corresponding to positions 3205–3223 and 4272–4290, respectively, of mDicer (NM_148948.1) relative to the start codon. The siRNAs were deprotected according to the manufacturer's instructions, and annealed as previously described (Elbashir et al., 2001). EGFP and mDicer dsRNA were prepared as previously described (Stein et al., 2003a); the mDicer dsRNA corresponded to nucleotides 33–1521 (GenBank accession number AF43085).

Results

Expression of transposable elements during preimplantation development

Repetitive sequences exhibit increased expression during the major phase of genome activation, which occurs during the 2-cell stage (Ma et al., 2001). The presence of RNAi during preimplantation development suggests that one role of RNAi could be to constrain expression of repetitive sequences. Before testing this hypothesis, we compared the abundance of repetitive sequences (most of which are derived from mobile elements) among cDNA libraries prepared from different preimplantation embryo stages (Ko et al., 2000). These data revealed an increase in abundance of repetitive sequences at the 2-cell stage (Fig. 1, open bars) when the zygotic genome activates (Schultz, 2002) and several mobile elements were highly expressed. It was possible that a biased cDNA library preparation distorted the actual representation of repetitive sequences in the transcriptome of the preimplantation embryo. A several-fold increase, however, in the abundance of mobile sequences in mouse 2-cell embryos was also observed when two other unamplified cDNA libraries (the Knowles and Solter 2-cell embryo and blastocyst cDNA libraries; (Rothstein et al.,

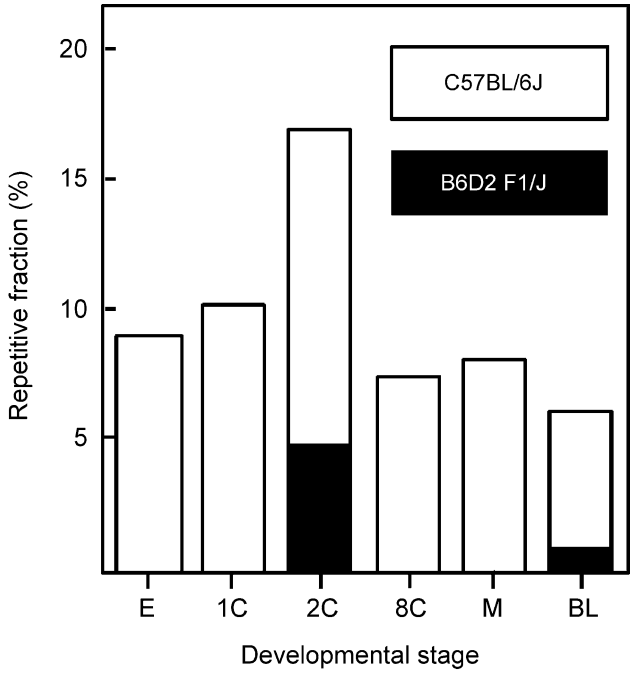

Fig. 1. Relative abundance of repetitive sequences in cDNA libraries from preimplantation embryos. Open bars, abundance of repetitive sequences in cDNA libraries from ERATO-DOI sequencing project (Ko et al., 2000). Solid bars, estimated abundance of transposable element sequences based on analysis of Unigene libraries storing the Knowles and Solter 2-cell and blastocyst cDNA libraries from Genbank. A difference in mouse strains used to generate these libraries is indicated on the right. E, metaphase II-arrested egg; 1C, 2C, 8C, 1-cell, 2-cell, and 8-cell stage embryo, respectively; M, morula; BL, blastocyst.

1992) were compared (Fig. 1, solid bars). We focused our subsequent studies on MuERV-L and IAP, two autonomous LTR (long terminal repeat) retrotransposons (Fig. 2A), because our analysis of the cDNA libraries revealed that their transcripts are highly abundant in the preimplantation embryo.

IAP is one of the most aggressive parasitic sequences known in the mouse genome and present in approximately 1000 copies per haploid genome (Kazazian and Moran, 1998; Kuff and Lueders, 1988). IAP is expressed in early embryos (the blastocyst contains approximately 150,000 transcripts (Pikó et al., 1984)), some normal tissues and certain transformed cell lines (Kuff and Lueders, 1988; Lueders and Kuff, 1977), while its expression in other cells is repressed by DNA methylation (Walsh et al., 1998).

MuERV-L (Benit et al., 1997) is a recently discovered endogenous retrovirus that belongs to the family of ERV-L elements present in all placental mammals. Three predicted bursts of expansion in the last few million years have resulted in an estimated 100–200 copies of MuERV-L in the mouse genome (Benit et al., 1999). The number of MuERV-L sequences in the mouse genome is actually much higher than that. There are several hundred longer sequences, most of which carry various deletions, and many more short (mostly LTR) sequences (Svoboda, unpublished observation). Initial studies of MuERV-L sequences concluded that it may be still an active element because of its sequence conservation and intact ORFs (Benit et al., 1999; Khier et al., 1999). The most recent study using the public mouse genome sequence reached a similar conclusion (Costas, 2003). That study identified 38 full-length MuERV-L insertions, of which 22 maintained intact ORFs, and in 16 of these, both LTRs were identical. Furthermore, there is a report of an MuERV-L insertion into the mouse *Hdac1* gene observed only in the 129SV strain of mice suggesting that MuERV-L was recently active (Khier et al., 1999). Analysis of cDNAs deposited in the dbEST database in the Genbank indicates that MuERV-L is transiently expressed during the 2-cell stage and then repressed to very low levels. We previously identified MuERV-L as a transcript whose repression is relieved in early embryos by inducing histone hyperacetylation, suggesting that the repression is mediated at the level of chromatin structure (Ma et al., 2001). Of particular interest is that MuERV-L mRNA is probably one

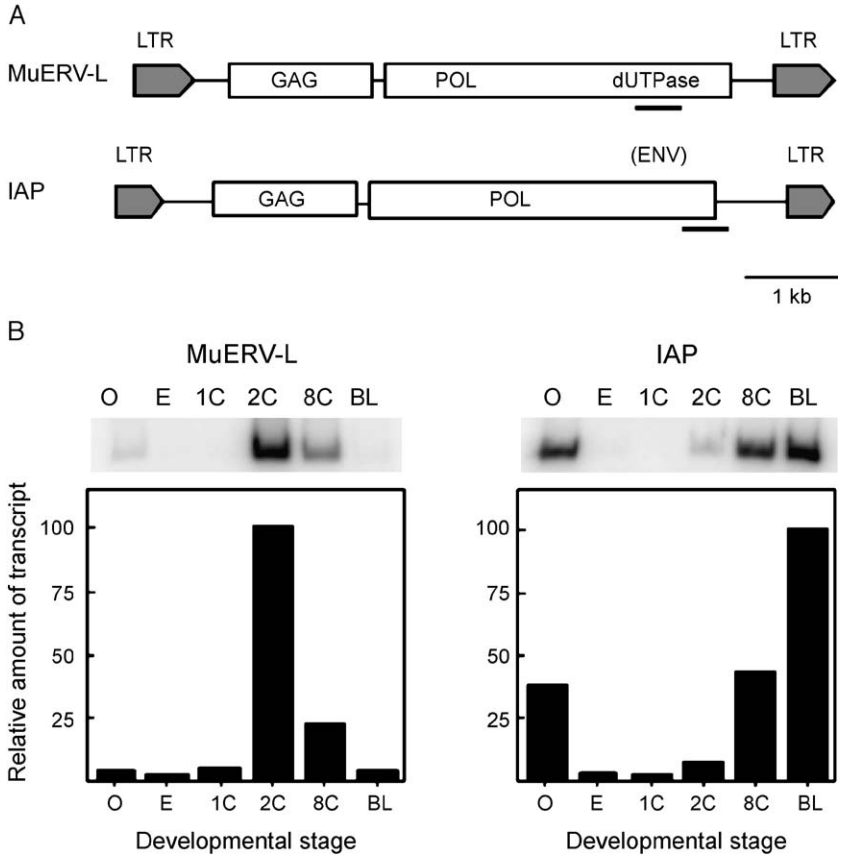

Fig. 2. Analysis of retrotransposons MuERV-L and IAP. (A) Schematic organization of MuERV-L and IAP based on Genbank annotation. LTR, long terminal repeat; GAG, similarity to retroviral gag genes (group-specific-antigen genes); POL, reverse transcriptase; ENV, envelope protein. A line at the 3′ end of each element indicates the region used in the RT-PCR analysis of expression of these elements. (B) Temporal pattern of MuERV-L and MuERV-L mRNA expression. The upper panel shows phosphorimager scans of one representative experiment and the graphs depict the quantification of these results, which are expressed relative to the stage in which the expression is highest. Each lane represents a signal from five embryo equivalents. O, fully grown germinal vesicle-intact oocyte. E, 1C, 2C, 8C, BL are as described in the legend to Fig. 1.

of the most abundant polyA RNAs found at the 2-cell stage according to the Unigene database.

We used RT-PCR to analyze the temporal pattern of expression of these two retroelements during preimplantation development (Fig. 2B). Expression of MuERV-L displayed a transient increased expression during the 2-cell stage, which is consistent with the data in dbEST and Unigene databases and a recent report (Wang et al., 2001). Following degradation of maternal IAP transcripts, zygotic expression was observed in the 2-cell embryo and IAP transcripts accumulated steadily increased throughout preimplantation development. Interestingly, expression of L1 retroelement, which belongs to the LINE class of retroposons, also initiated during the 2-cell stage and progressively increased with preimplantation development (data not shown).

IAP and MuERV-L express both sense and antisense RNA strands in the early embryo

Although neither IAP nor MuERV-L use a dsRNA intermediate during their natural life-cycle, there are numerous ways in which these elements can generate dsRNA intermediates that could trigger the RNAi response, for example, by bidirectional promoter activity of their LTRs or internal inversions (Waterhouse et al., 2001). In fact, IAP and L1 dsRNA have been isolated from liver cells (Kramerov et al., 1985). Because it was not feasible to isolate and analyze dsRNA from preimplantation embryos directly, we examined if we could detect expression of the antisense RNA strand from IAP and MuERV-L by RT-PCR (Fig. 3A). Total RNA from 250 2-cell or 8-cell embryos was split into five groups and four of these groups were reverse-transcribed using specific primers for sense and antisense strands of IAP and MuERV-L. The last group was used for a minus RT control. Subsequent RT-PCR analysis detected the presence of cDNAs derived from sense and antisense RNA strands in 2-cell and 8-cell embryos (Fig. 3B). Although amplification of antisense-derived cDNA suggested lower levels (5–40% of sense RNA, depending on the number of PCR cycles), these apparent differences may include the effects of different efficiencies of RT priming of different primers.

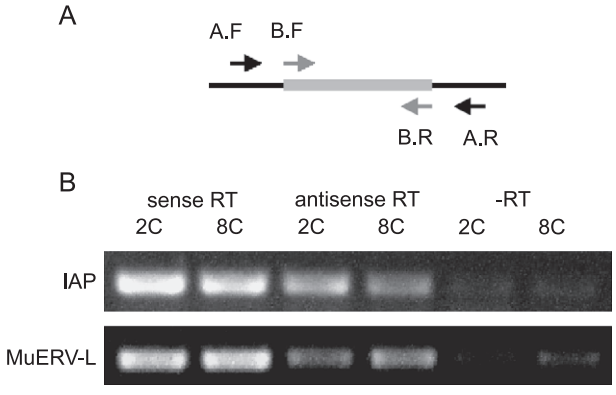

Fig. 3. RT-PCR detection of sense and antisense RNA strands from IAP and MuERV-L. (A) Relative position of primers used in this experiment. Reverse transcription was primed with either A.F or A.R primer specific for each element and the reverse-transcribed cDNA was amplified (grey fragment) using the inner pair of B primers (for primer sequences and conditions see Table 1). (B) Detection of both RNA strands in 2-cell and 8-cell embryos. An ethidium bromide-stained 1.3% agarose gel is shown. 2C, 2-cell embryo; 8C, 8-cell embryo; antisense RT, antisense strand reverse transcription and amplification; sense RT, sense strand reverse transcription and amplification; −RT, minus RT control. The experiment was performed twice and similar results were obtained in each case. Shown are the results of one experiment.

It was formally possible, however, that the RT-PCR amplification of the antisense RNA was the result of mis-priming in the RT reaction. For example, if a primer specific for the antisense RNA strand would hybridize nonspecifically to the sense RNA downstream of the amplified region, the RT-PCR amplification would detect it as reverse-transcribed antisense RNA. We excluded this scenario by demonstrating that the antisense RNA amplifying primer could not initiate reverse transcription on the sense strand; if the primer could initiate such reverse transcription, a PCR reaction with a single primer should yield a band of the size corresponding to the distance between the mis-priming site and the original primer position. Because the analyzed region is localized close to the 3′ end of the mRNA of transposable elements, the maximum size of the possible product should be approximately 2 kb. The absence of PCR products under conditions that permit generating long products suggested that the RT-PCR-based detection of antisense RNA was specific.

Inhibition of RNAi by mDicer siRNA microinjection

To study a potential relationship between RNAi and expression of IAP and MuERV-L, we first tested how efficiently we could inhibit RNAi by microinjecting siRNA against mDicer. In this experiment, we used Oct-4 promoter-driven EGFP expression in preimplantation embryos as a model system. One-cell embryos were microinjected with EGFP dsRNA or mDicer siRNA + EGFP dsRNA. Injected embryos were cultured until the 8-cell stage when the EGFP fluorescence was evaluated. As anticipated, EGFP dsRNA-injected embryos displayed an approximately 95% reduction in fluorescence when compared to uninjected controls (Figs. 4A and B). In contrast, embryos injected with both EGFP dsRNA and mDicer siRNA revealed levels of fluorescence substantially greater than that observed in EGFP dsRNA-injected embryos (Figs. 4B and C); the fluorescence levels in these embryos were approximately 40% that of uninjected controls (Figs. 4A and C). Thus, injection of mDicer siRNA inhibited the RNAi pathway by approximately 60% ($P < 0.001$, ANOVA followed by Bonferroni's multiple comparison test).

siRNAi can enter RISC (RNA-induced silencing complex) independently of being processed from dsRNA by dicer (Elbashir et al., 2001; Martinez et al., 2002). It was possible that the RNAi pathway directed toward targeting

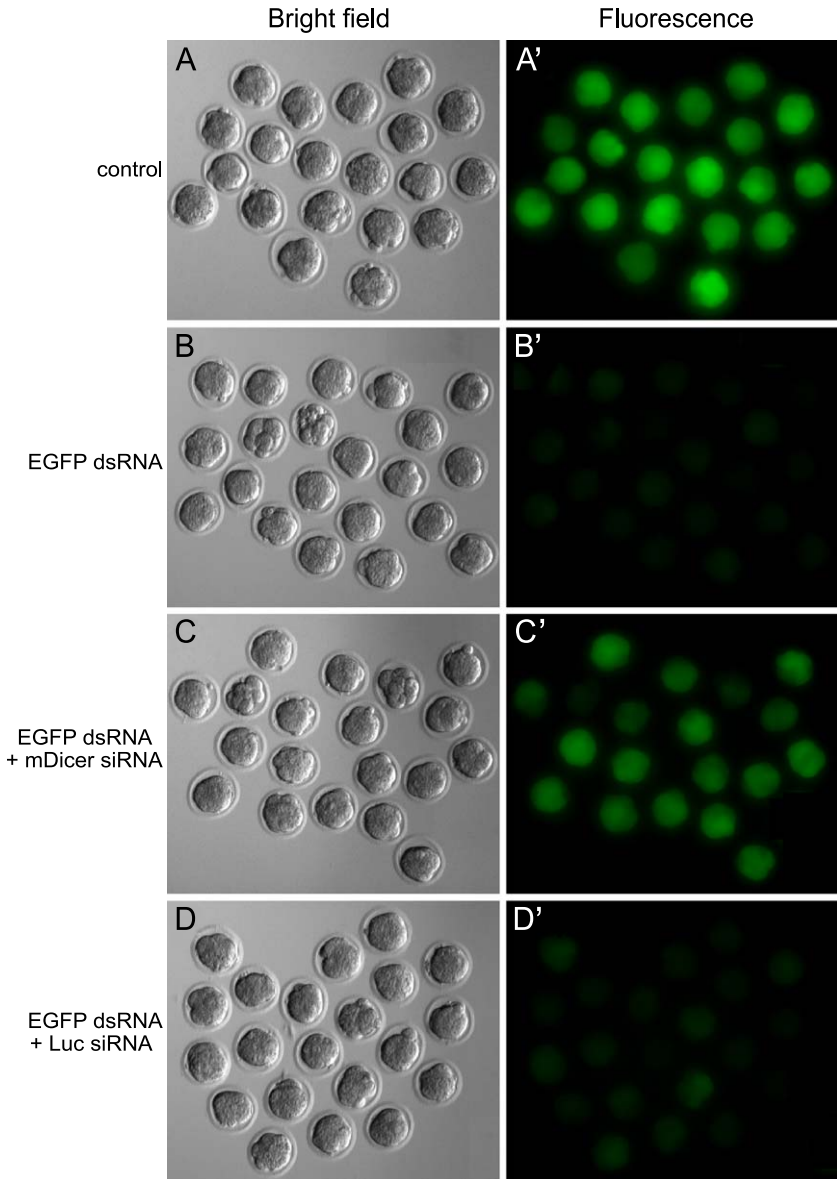

Fig. 4. Inhibition of RNAi by mDicer siRNA microinjection. One-cell transgenic embryos that express an Oct4 promoter-driven EGFP transgene were (A) cultured to the late 8-cell/early morula stage, or cultured to the late 8-cell/early morula stage following injection of 1-cell embryos with (B) EGFP dsRNA, (C) EGFP dsRNA and mDicer siRNA, or (D) EGFP dsRNA and Luc siRNA. The experiment was performed twice and similar results were obtained in each case; approximately 20 embryos in each treatment group were analyzed for each experiment.

specific mRNAs could be inhibited by saturating the RISC with "irrelevant" siRNAs, that is, the mDicer siRNA effect was not due to targeting mDicer mRNA. To test this hypothesis, 1-cell embryos that express Oct-4-driven EGFP were injected with EGFP dsRNA and luciferase (*Luc*) siRNA, and fluorescence was examined at the 8-cell/morula stage (Fig. 4D). Results of these experiments indicated that the level of fluorescence was reduced by approximately 93% relative to uninjected embryos (Fig. 4D); this decrease was not statistically different than that observed following injection of EGFP dsRNA (Fig. 4B). Therefore, the inhibitory effect of mDicer siRNA can be solely accounted for by mDicer inhibition of the RNAi pathway.

Inhibition of RNAi correlates with higher levels of expression of MuERV-L and IAP sequences

If expression of MuERV-L and IAP sequences is constrained by an RNAi mechanism, inhibiting RNAi should lead to their increased expression. Therefore, we assayed by semi-quantitative RT-PCR the relative abundance of IAP and MuERV-L mRNA in 8-cell embryos in which RNAi was inhibited as described above by microinjecting 1-cell embryos with siRNA against mDicer. We observed a 50% increase in expression of IAP and MuERV-L relative to controls (Fig. 5A); similar results were obtained when embryos were injected with mDicer dsRNA targeting a

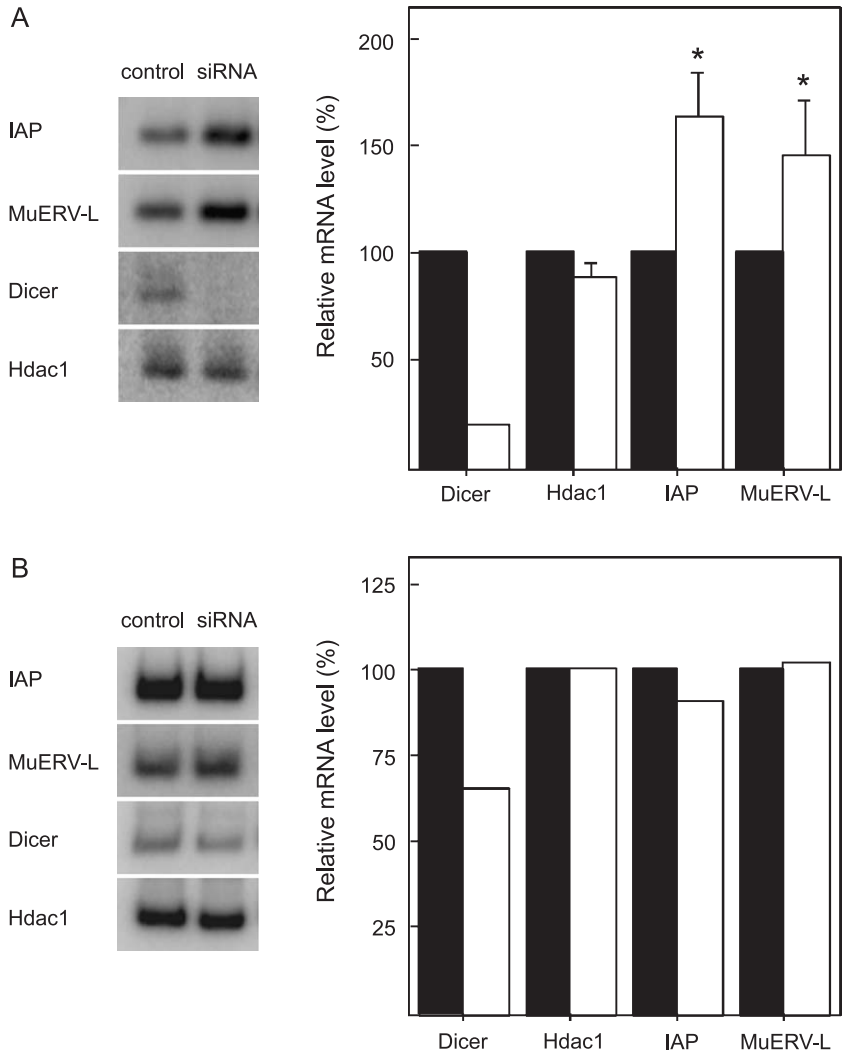

Fig. 5. Effect of inhibiting RNAi by targeting mDicer on IAP and MuERV-L expression. (A) One-cell embryos were microinjected with mDicer siRNA or long dsRNA, cultured to the 8-cell stage and analyzed by semiquantitative RT-PCR. The left panel shows portions of a phosphorimager scan from a representative experiment. The graph depicts the quantification of the results of five independent experiments, which included three experiments using siRNA and two experiments using long dsRNA; similar results were obtained using either approach. Solid bars, uninjected embryos; open bars, injected embryos. The data were normalized to the control uninjected 8-cell embryos and are expressed as the mean ± SEM. *, the difference compared to the control is significant, $P < 0.02$, t test. (B) Inefficient mDicer inhibition does not have any effect on MuERV-L and IAP mRNA levels.

different region of mDicer mRNA. The effect was specific because mDicer mRNA was reduced >80%, whereas there was no decrease in the abundance of the nontargeted HdAc1 (Fig. 5A) or histone H1A transcripts (data not shown). Moreover, there was no significant increase in either MuERV-L or IAP expression following microinjection of either a EGFP dsRNA or an EGFP siRNA (data not shown).

Lastly, one of the siRNA microinjection experiments resulted in only a very modest reduction in mDicer mRNA that we subsequently deduced was due to a partial loss of siRNA activity. In this experiment, there was no effect on MuREV-L and IAP mRNA levels (Fig. 5B). Thus, conditions in which mDicer mRNA was not effectively targeted did not result in enhanced MuREV-L and IAP mRNA abundance. This observation served as another control, albeit an unintentional one, for the specificity of the effect of siRNA microinjection on MuREV-L and IAP mRNA levels. Taken together, these data suggest that RNAi constrains expression of retrotransposon-derived repetitive sequences during preimplantation development.

Discussion

The major phase of genome activation occurs during the 2-cell stage of the preimplantation mouse embryo and is coupled with extensive remodeling of chromatin structure due to the protamine–histone exchange and DNA replication (Schultz, 2002). Repetitive elements seemingly exploit this opportunity for expression because they represent 5–15% of cloned cDNAs from the 2-cell embryo (our EST

analysis and (Ma et al., 2001)). Moreover, the global DNA demethylation that occurs during preimplantation development (Mayer et al., 2000; Oswald et al., 2000; Santos et al., 2002), except for imprinted genes that maintain their differential methylation pattern (Oswald et al., 2000), may make preimplantation embryos more susceptible to negative effects of sequences whose expression is normally repressed by DNA methylation.

Retroelements can generate dsRNA by a variety of mechanisms (Waterhouse et al., 2001) and in fact, we detect expression of antisense sequences to both MuERV-L and IAP that could form dsRNA to trigger the RNAi pathway. Expression of either long hairpin dsRNA from a transgene (Stein et al., 2003b) or expression of both sense and antisense RNA strands can elicit RNAi (Wang et al., 2003). It is likely that enough MuERV-L dsRNA is expressed in the 2-cell embryo to trigger RNAi. Two-cell embryos would contain 10^5 MuERV-L transcripts, based on 10 pg of mRNA in the 2-cell embryo (Clegg and Pikó, 1983), and assuming an average mRNA size of 2.5 kb and that MuERV-L constitutes approximately 2% of the total mRNA (using the MuERV-L frequency in the 2-cell Knowles and Solter Unigene Library 88). If the antisense RNA composes only 1% of this amount, then approximately 10^3 molecules of dsRNA could be present. Ten thousand molecules of dsRNA promote a robust RNAi effect in mouse oocytes, while the effect is not observed with 10^2 molecules (Svoboda et al., 2000). Thus, 10^3 molecules likely can trigger RNAi in the oocyte.

Partially inhibiting RNAi by specifically destroying mDicer mRNA by mDicer siRNA or long dsRNA leads to a selective 50% increase in expression of MuERV-L and IAP repetitive sequences in 8-cell embryos. This increase likely corresponds to tens of thousands of additional transcripts. For example, the 8-cell embryo would contain approximately 40,000 MuERV-L transcripts (approximately 25% of that present in the 2-cell embryo (see aforementioned calculation and Fig. 2B) and approximately 60,000 IAP transcripts (40% of that in the blastocyst, Fig. 2B). Our experimental design limits the reduction in mDicer mRNA (and presumably mDicer activity) due to the presence of maternal mDicer protein (Svoboda and Schultz, unpublished observations). Moreover, targeting of mDicer mRNA occurs continuously following injection of 1-cell embryos up to the 8-cell stage at which point MuERV-L and IAP transcript abundances were measured. Thus, the increase in MuERV-L and IAP transcript abundances might even be greater if mDicer could be reduced to greater extent, for example, eliminating maternal mDicer.

We did not observe any apparent effect on development to the blastocyst stage of 1-cell embryos injected with either dicer siRNA or dsRNA (Svoboda, Stein, and Schultz, unpublished observations). This is not surprising because developmental failure of *Dicer1*-null mice occurs following implantation (Bernstein et al., 2003), maternal dicer perhaps providing sufficient stores to support development beyond implantation. Thus, any early physiological impact of increased expression of repetitive elements would likely be minimal given the low transposition frequency. Moreover, if the retrotransposition frequency is increased, the retrotransposition event would presumably occur in a single blastomere. Unless the event disrupted an essential gene critical for development, for example, cell proliferation (and would also be haploid insufficient), it is most unlikely that a readily observable phenotype would be observed by the blastocyst stage.

We propose that, in addition to a transcriptionally repressive mechanism(s), the preimplantation embryo also exploits a post-transcriptional mechanism, namely RNAi, to constrain the opportunistic expression of repetitive sequences that could, eventually, result in their further genomic expansion. Superimposed on genome activation during the 2-cell stage is the development of a chromatin-mediated transcriptionally repressive state that is relieved by inducing histone hyperacetylation in 2-cell embryos (Schultz, 2002). Inducing histone acetylation in 2-cell embryos prevents the decrease in expression of repetitive elements that occurs between the 2- and 4-cell stages (Ma et al., 2001). Increased IAP expression correlates with decreased DNA methylation commencing around the 8-cell stage (Howlett and Reik, 1991), and de novo DNA methylation that occurs following blastocyst formation (Santos et al., 2002) is coupled to the repression of IAP expression because there is a dramatic increase in IAP expression in *Dnmt1* null mice (Walsh et al., 1998). Although development of these transcriptional mechanisms represses expression of these repetitive elements, they do not address how the embryo copes with sequences that are already expressed. One function of RNAi may therefore be to target destruction of these transcripts and hence constrain expression of repetitive sequences. If some of these transcripts are transposition competent and if retrotransposition can occur in the preimplantation embryo, RNAi may therefore protect genomic integrity by constraining retrotransposition.

Finally, components of the RNAi machinery may also modulate retrotransposon expression at the level of chromatin. Two recent studies in the fission yeast imply that dsRNA arising from centromeric repeats targets formation and maintenance of heterochromatin through RNAi (Hall et al., 2002; Volpe et al., 2002). A centromere of the fission yeast is composed of a nonrepetitive AT-rich core flanked by several classes of centromeric repetitive elements that bind to a retrotransposon homolog (Clarke et al., 1986; Fishel et al., 1988). Similarly, mammalian centromeric regions are also enriched in transposable element sequences (Barry et al., 1999). If a similar connection between heterochromatin and RNAi exists in mammals, RNAi might also direct transcriptional silencing of repetitive sequences during preimplantation development, and thus also play a role in establishing the transcriptionally repressive state that develops following genome activation.

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