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Dicer-Dependent Turnover of Intergenic Transcripts from the Human β-Globin Gene Cluster

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The widespread occurrence of intergenic transcription in eukaryotes is increasingly evident. Intergenic transcription in the β-globin gene cluster has been described in murine and human cells, and models for a role in gene and chromatin activation have been proposed. In this study, we analyze intergenic transcription and the chromatin state throughout the human β-globin gene cluster and find that the data are not consistent with such activation-linked models. Thus, intergenic transcript levels correlate with neither chromatin activation nor globin gene expression. Instead, we find that intergenic transcripts of the β-globin gene cluster are specifically upregulated in Dicer-deficient cells. This is accompanied by a shift towards more activated chromatin as indicated by changes in histone tail modifications. Our results strongly implicate RNA interference (RNAi)-related mechanisms in regulating intergenic transcription in the human β-globin gene cluster and further suggest that RNAi-dependent chromatin silencing in vertebrates is not restricted to the centromeres.

The human β-globin gene cluster spans over 70 kb on chromosome 11 consisting of five β-globin-like genes. It is regulated by an upstream locus control region (LCR) which confers high-level and tissue-specific expression on linked globin genes in transgenic mice (18). Although the LCR autonomously opens chromatin, presumably by recruiting chromatin remodeling complexes through transcription factor association, a region upstream of the LCR has been inferred to modulate β-globin locus chromatin as well (21, 36). This region consists of a number of repetitive elements, one of which is an endogenous retroviral (ERV) solitary long terminal repeat (LTR) (ERV solo LTR). This element has been identified as a major promoter of intergenic transcription in both erythroid cells and nonerythroid HeLa cells following treatment with the histone deacetyltransferase inhibitor trichostatin A (TSA) (35).

The presence of intergenic transcription in the human β-globin gene cluster has given rise to various models trying to explain their existence (reviewed in reference 43). All of these models essentially positively link intergenic transcription to globin gene activity. The notion that intergenic transcription follows the same 5′-3′ polarity as the structural globin genes (subsequently referred to as "sense" orientation) (2) triggered speculation that intergenic transcription may be a means of delivering polymerases to the globin gene promoters during gene activation and development (30). A variation of this tracking model explains the spatially and temporally coordinated activation of globin genes during development by arguing that intergenic transcription would be necessary for opening chromatin at the appropriate developmental stages, thus facilitating globin gene expression (16). Intergenic transcription should therefore be active at all erythropoietic stages in the LCR, consistent with its role as a constitutive globin enhancer, while it should be coupled to resident globin gene activity in the embryonic-fetal and adult globin domains. Importantly, a chromatin boundary was identified between the γ- and β-globins. This appeared to coincide with the upstream border of an intergenic transcription domain spanning the adult globin genes, consistent with developmentally regulated chromatin activation by intergenic transcription (16).

Based on comparative intergenic transcript and chromatin analysis in the context of both ε/γ-globin and β-globin gene expression, we find that no such predicted positive correlation exists between intergenic transcript abundance and open chromatin and/or globin gene expression. This and the presence of both sense and antisense transcripts prompted us to instead investigate the involvement of RNA interference (RNAi) in the regulation of intergenic transcripts across the β-globin gene cluster. We show that intergenic transcripts are indeed specifically upregulated in cells knocked down for Dicer. Adding to the mounting evidence that RNAi/Dicer-related processes are also important in determining silent chromatin in vertebrates (15, 25, 27, 31), our results suggest that Dicer knockdown promotes chromatin activation at the noncentromeric β-globin locus. We propose a model in which intergenic transcription mediates the formation of silent chromatin in the absence of erythrocyte-specific transcription factors.

MATERIALS AND METHODS

Cell culture. HeLa cells were grown in Dulbecco's modified Eagle's medium with 2 mM glutamine and 10% fetal calf serum. Dicer knockdown (23) was modified as follows: cells were split 1 day after the first small interfering RNA (siRNA) treatment and then treated again the next day. TSA induction was performed as described previously (35). Phase II day 13 adult erythroid cells (11) were a generous gift from W. G. Wood (IMM, Oxford, United Kingdom). K562 cells were cultured in RPMI 1640 medium with 2 mM glutamine and 10% fetal calf serum. In the globin induction experiments, 0.6 mM butyrate or 30 μM hemin (prepared according to the method described previously in reference 12) was applied for 68 h.

RT-PCR. Total RNA was isolated with Trizol reagent (Invitrogen). Genomic DNA-free RNA was obtained by two acid phenol-chloroform extractions and DNase I treatment followed by a final acid phenol-chloroform extraction. First-strand cDNA was obtained with Superscript III reverse transcriptase (RT) (Invitrogen) and a QIAquick PCR purification kit (QIAGEN) for use in PCR. In

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TABLE 1. Nuclear-cytoplasmic RNA fractionation of TSA-treated HeLa cells by real-time RT-PCR

Amplicon	Fractionation (relative units)	
	Control siRNA	Dicer knockdown
"ERV down" cytoplasmic	None	None
"ERV down" nuclear	3	13.7
"γ-Unspliced" cytoplasmic	None	None
"γ-Unspliced" nuclear	0.44	1.9
"γ-Spliced" cytoplasmic	9.9	10.5
"γ-Spliced" nuclear	15.1	15.1

qualitative RT-PCR analyses, PCRs were carried out within the linear range of amplification. Quantitative PCR was performed with a Rotorgene 3000 real-time PCR machine (Corbett Research) using QuantiTect SYBR green chemistry (QIAGEN). Each PCR was done three to four times with standard deviations from the mean varying no more than 20%. Melting curve analyses confirmed that only one product was amplified. Controls omitting reverse transcriptase indicated insignificant genomic DNA carryover. Control and knockdown samples were normalized for actin when endogenous transcript levels were measured. For comparative quantitation between probes in a given sample, the same genomic DNA was used for the standard curve for all primer pairs except for the use of cDNA for measuring the abundance of spliced transcript.

To relate the amount of spliced transcripts to that of intergenic transcripts, the corresponding unspliced transcripts were quantified using both genomic DNA as well as a plasmid containing equimolar copies of spliced and unspliced templates. Thus, the level of spliced transcripts can be related to the level of intergenic transcripts via the abundance of unspliced transcript. Only spliced product was amplified in reactions spanning the long (~850-nucleotide) second introns as verified by agarose gel and melting curve analyses.

To calculate the amount of a given transcript per nuclear and cytoplasmic equivalent in the RNA fraction analyses, we determined how much total and nuclear RNA was isolated from the same number of cells. As intergenic and unspliced genic transcripts were not detected in the cytoplasm (Table 1), we assumed that there was no loss of nuclear RNA to the cytoplasmic fraction. After subtraction of the nuclear RNA from total RNA, ~72% of the total RNA was determined to be cytoplasmic. This value increases to ~85% considering that ~20% of total mitochondrial cytochrome oxidase II message was detected in the nuclear fraction (see Fig. 6C). β-Globin cluster primer pair coordinates and non-β-globin primers are available at http://www.path.ox.ac.uk/dirsci.htm #proudfoot.

Nuclear-cytoplasmic fractionation. HeLa and K562 cells were resuspended in lysis buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 0.5% NP-40) on ice for 10 min. The reaction mixture was then underlayered with an equal volume of lysis buffer containing 12% sucrose and spun for 10 min at 15,800 × *g*. The supernatant (cytoplasmic fraction) was acid phenol-chloroform extracted twice and precipitated with ethanol. RNA from the pellet (nuclear fraction) was isolated with Trizol reagent. Cytomegalovirus (CMV)–β-globin–PTB plasmid is derived from CMV–β-globin (7) with a fragment containing the PTB siRNA target site (46) inserted into the BamHI site of human β-globin exon 2. RNA from these experiments was harvested 16 h posttransfection. At this time, PTB protein activity is not appreciably affected by RNAi treatment (46).

ChIPs. Chromatin immunoprecipitation assays (ChIPs) were performed as described previously (14), with the following modifications. A total of 5×10^6 and 15×10^6 cells were fixed in 1% formaldehyde for 10 min. Chromatin was sheared to an average size of 0.5 to 1 kb, and the immunoprecipitated DNA was recovered using the QIAquick PCR purification kit (QIAGEN). Quantification by real-time PCR was performed as described above. Standards were obtained from one of the input DNA samples. Comparisons were only made for samples processed in parallel after normalization to input. Histone H4 acetylation ChIPs were performed more than three times, histone H3 lysine 4 (H3K4) dimethylation ChIPs were performed three times, and H3K9 acetylation ChIPs were performed twice, each with similar results.

siRNAs. siRNAs were purchased from QIAGEN (ε-globin siRNA) and Dharmacon (all other siRNAs). Dicer siRNA duplex targets 5′-AC TGC TTG AAG CAG CTC TGG A-3′ (P-002010-01-20) (1); control siRNAs used were "nonspecific control duplex IX" (D-001206-09-20; used in all RNAi experiments) and "Cy3-luciferase GL2 duplex" (D-001110-01-20; used in addition in histone H4 acetylation and H3K4 dimethylation ChIPs); ε-globin siRNA targets 5′-CTT CCT TTG GAG ATG CTA TTA-3′ of ε-globin exon 2. PTB RNAi was previously described (7).

Antibodies. Abcam antibody ab2380 recognizes all acetylated forms of histone H4 but binds best to tri- and tetra-acetylated H4; Upstate antibody 07-030 is specific for dimethylated lysine 4 of histone H3; Upstate antibody 07-352 is specific for acetylated lysine 9 of histone H3; monoclonal antibody Sigma T 5168 detects human α-tubulin; and the Dicer-specific antibody was a gift from W. Filipowicz (6) (FMI, Basel, Switzerland).

RESULTS

β-Globin intergenic transcript profiles: sense and antisense transcripts. We wished to complement previous nascent intergenic transcription analysis (2) by obtaining a quantitative profile of intergenic transcripts throughout the human β-globin gene cluster. We therefore measured steady-state transcript levels from cells expressing either ε/γ- or β-globin by real-time RT-PCR (Fig. 1A and B). Late-stage erythroid cells were obtained from adult peripheral blood erythroid progenitor cells according to previously published procedures (11). Such cells show a distinctive profile (Fig. 1A). As expected, β-globin accounted for most of the spliced genic transcripts at this stage, with some γ-globin (47) and comparatively negligible amounts of ε-globin. Note that the transcript levels are shown on a logarithmic scale. The typically more than 1,000-fold-higher transcript levels of (spliced) β-globin compared to intergenic transcripts are probably attributable to differential stabilities, given that actual transcription rates, based on nuclear run-on analysis, did not differ by more than 10-fold (2; K. E. Plant and N. J. Proudfoot, unpublished observations). Unexpectedly, antisense as well as sense intergenic transcripts were detected.

Sense strand intergenic transcripts were of low abundance upstream of the ERV element (ERV up 1 and 2). This was followed by a local peak associated with the ERV element (ERV in, ERV down) and notably weaker levels over the LCR (HS5 down, HS 2/3). This is in contrast to models that link intergenic transcription to chromatin activation which would predict such transcripts to be relatively abundant in the LCR. Antisense transcript abundance, meanwhile, exceeded that of sense upstream of ERV (ERV up 1 and 2) and just downstream of HS 5 (HS5 down).

Equally unexpected was the fact that the intergenic transcript profile in adult erythroid cells showed a peak ~5 kb downstream of the fetal γ-globin genes (γ-Alu), i.e., well upstream, ~7 kb, of the previously proposed boundary of a downstream intergenic transcription domain of adult erythropoiesis (16). As this transcript peak is also observed in K562 cells (Fig. 1B), this element appears to be generally active in erythroid cells, independent of which globin gene is expressed. The high levels of apparently intergenic transcripts just up- and downstream of the structural β-globin gene are likely attributable to premature transcription initiation (29) and incomplete transcription termination (10), respectively.

Although K562 cells are of erythroleukemic origin, which may limit their utility in certain studies, they should be informative for studying intergenic transcription in the context of embryonic ε- and γ-globin expression (Fig. 1B). Such cells are likely to have arisen from a transformation event in the stem cell compartment (17), a view that is supported by their ability to differentiate with erythrocyte-, megakaryocyte-, and monocyte-like properties following drug treatment (34). Similar to

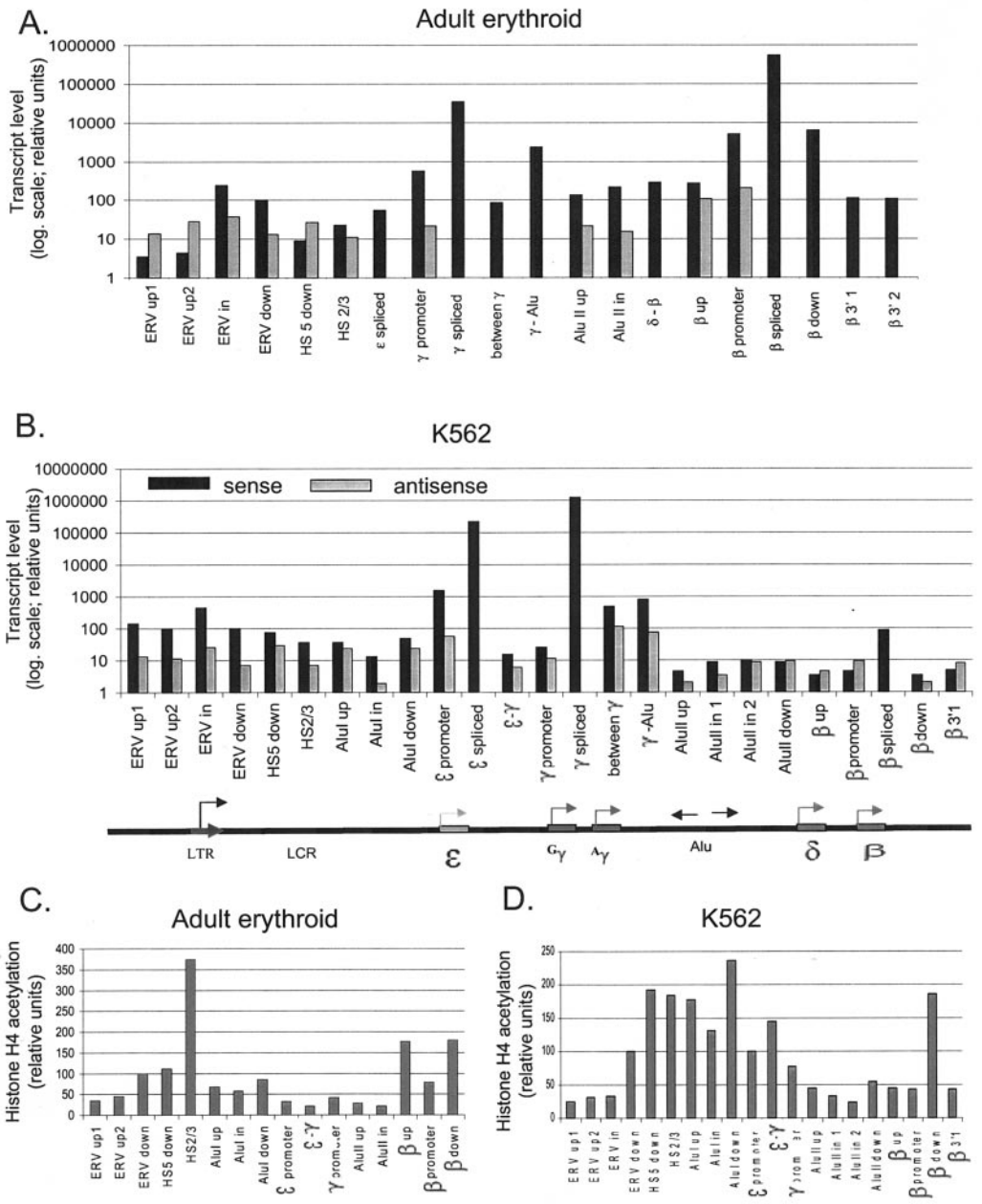

FIG. 1. β-Globin cluster transcript and chromatin profiles. (A) Adult erythroid cell (Fibach culture) transcript profile (real-time RT-PCR). Both sense (dark bars) and antisense (light bars) intergenic transcripts are shown on a logarithmic scale. (B) K562 transcript profile (real-time RT-PCR). Most of the data shown in panels A and B have been reproduced at least once to verify the reproducibility of the profiles (data not shown). (C) Histone H4 acetylation of adult erythroid cells (ChIP by real-time PCR). (D) K562 histone H4 acetylation (ChIP by real-time PCR). A schematic of the β-globin gene cluster is aligned below panel B to indicate the relative location of an amplicon. Suffixes "up" and "down" indicate a position 5′ and 3′, respectively, to the named element; the absence of a bar for "antisense" indicates that "antisense" was not measured at this element.

adult erythroid cells, the levels of the predictably abundant ε- and γ-globin transcripts far exceed that of the intergenic transcripts. Again, there are antisense transcripts detected in addition to sense transcripts. These tend to be less abundant than their sense counterparts but reach similar levels in the downstream half of the cluster which is transcriptionally less active. In contrast to intergenic transcripts in adult erythroid cells, intergenic transcripts in K562 are generally more abundant in the upstream half of the cluster and are highest upstream and within the ERV element.

Intergenic transcript profiles are inconsistent with a role in chromatin activation. To test whether intergenic transcript levels are positively correlated with open/active chromatin as predicted by the chromatin activation model, levels of histone H4 acetylation, a marker of activated chromatin, were measured across the cluster by ChIP. Related studies on the mouse

and human cluster previously indicated that histone acetylation was organized in developmentally regulated domains (14, 39); to our knowledge, however, no comprehensive and direct comparison of β-globin locus histone acetylation had been undertaken between ε/γ- and β-globin-expressing human erythroid cells. As expected from its association with a number of DNase I-hypersensitive sites (44), this modification is highest in the LCR in both adult and K562 cells (Fig. 1C and D). The upstream ERV element apparently coincides with a chromatin boundary, as histone acetylation is significantly depleted further upstream. In agreement with previous findings (16), another chromatin boundary appears to coincide with Alu elements about 12 kb downstream of γ-globin. This is indicated by a trough in histone acetylation ("AluII up, AluII in" in Fig. 1C; "AluII up, AluII in [1, 2]" in Fig. 1D) and complementary high levels of histone H3K9 di- and trimethylation in both K562 and adult erythroid cells (see http://www.path.ox.ac.uk/dirsci.htm #proudfoot). These repetitive elements may play some role in the developmental regulation of globin gene expression as suggested by the deletional form of hereditary persistence of fetal hemoglobin in which their deletion is associated with persistent expression of fetal γ-globin during adult erythropoiesis (3, 8). Interestingly, there is increased histone acetylation further downstream in the transcriptionally relatively silent β-globin domain of K562, which may reflect a poised chromatin state (22) (Fig. 1B, "β spliced"). This, however, is not accompanied by a commensurate increase in intergenic transcripts. Moreover, inconsistent with activation-linked models of intergenic transcription, there are significant intergenic transcript levels upstream of ERV in K562 cells, whereas histone acetylation is quite low (compare Fig. 1B and D). There are also abundant intergenic transcripts in adult erythroid cells in the fetal globin domain which is similarly marked by low levels of histone H4 acetylation. Our findings therefore strongly argue against the view that intergenic transcription brings about chromatin activation (an alignment of intergenic transcript and histone acetylation levels is available at http://www.path.ox.ac.uk/dirsci.htm#proudfoot).

Intergenic transcripts are not increased following globin gene activation. We next wished to examine whether intergenic transcript levels are positively correlated with transcriptional activation of globin gene expression, as would be predicted if intergenic transcription was a means for recruiting RNA polymerases to globin promoters or if intergenic transcription was a side effect of strong nearby gene activity. We took advantage of the known property of hemin and butyrate to enhance the erythroid properties of K562 cells as manifested by the transcriptional induction of ε- and γ-globin genes (1, 9). As expected, spliced ε- and γ-globin transcripts were increased by five- to sixfold (Fig. 2A and B, side panels). Possibly related to its inhibitory effect on histone deacetylases, butyrate enhanced the levels of not only ε- and γ-globin but also spliced β-globin transcripts (Fig. 2B). Importantly, and in striking contrast to the robust induction of spliced globin transcripts, sense intergenic transcripts were not elevated following either drug treatment. Instead, it appeared to be slightly weakened at some elements, particularly in the upstream half of the cluster, while it was unchanged at other elements. Collectively, these data argue strongly against the view that intergenic transcription either is a side-product of genic transcription or is required to supply globin genes with polymerases for the activation of globin transcription.

Dicer knockdown substantially increases induced intergenic transcript levels. Intergenic transcription appears to be neither correlated with nor required for chromatin opening or gene activation. Furthermore, both sense and antisense intergenic transcripts are readily detectable. In light of mounting evidence that RNAi plays a significant role in the epigenetic organization of genomes from a range of organisms, including vertebrates (15, 25, 27, 31), we therefore tested the involvement of RNAi-related processes in regulating intergenic transcript levels in the β-globin gene cluster. Since silencing in this locus may be of particular importance in nonerythroid cells, we chose HeLa cells as a readily accessible model system. As such, intergenic transcription can be reversibly induced with the histone deacetylase inhibitor TSA in HeLa cells (35). Moreover, functional knockdown of Dicer with siRNAs had been demonstrated in this cell line previously (23).

Figure 3A demonstrates the potent induction of intergenic transcription by TSA for an element just downstream of the ERV element (ERV down), and as observed before for erythroid cells, not only sense but also antisense transcripts were induced. In addition to intergenic transcripts, TSA also induced genic transcripts (Fig. 3B). This is in contrast to previous nuclear run-on analyses that only detected intergenic transcription following TSA induction. This difference is likely accounted for by the greatly increased stability of spliced genic transcripts compared to that of nuclear-restricted intergenic transcripts. Regardless, these genic transcripts appear to be largely bona fide mRNA, as they are mainly spliced (Fig. 3B) and polyadenylated (see http://www.path.ox.ac.uk/dirsci.htm #proudfoot) and can be found in both the nucleus and cytoplasm (Table 1). Unspliced genic as well as intergenic transcripts, however, were restricted to the nucleus (Table 1). The physiological relevance of these weakly induced bona fide globin mRNAs is not immediately evident; however, it incidentally allowed us to learn more about the specificity with which RNAi accesses intergenic transcripts, as discussed below.

A series of knockdown experiments were then performed using Dicer siRNAs previously shown to abrogate Dicer function (23). RT-PCR and Western blot analyses confirmed that this siRNA efficiently depleted Dicer mRNA and protein (predicted size, 218 kDa), while actin mRNA and tubulin protein controls were unchanged (Fig. 4A and B). If Dicer was involved in turning over β-globin intergenic transcripts, then reduced Dicer activity would be predicted to lead to an increase in intergenic transcript levels. This was indeed the case. As demonstrated for the "ERV down" element, both sense and antisense transcripts were strongly upregulated in Dicer knockdown cells (Fig. 4C). Actin (spliced) transcript levels, however, were unchanged (Fig. 4A). Transcript levels were then profiled over the whole cluster in Dicer knockdown and control siRNA-treated HeLa cells following TSA induction (Fig. 4D; antisense data are available at http://www.path.ox.ac.uk/dirsci.htm#proudfoot). This confirmed that intergenic transcript levels were consistently upregulated in Dicer knockdown cells, especially around the upstream ERV element. Similar to K562, intergenic transcription appeared to be highest in this upstream half of the cluster, with decreasing intergenic transcript levels further downstream.

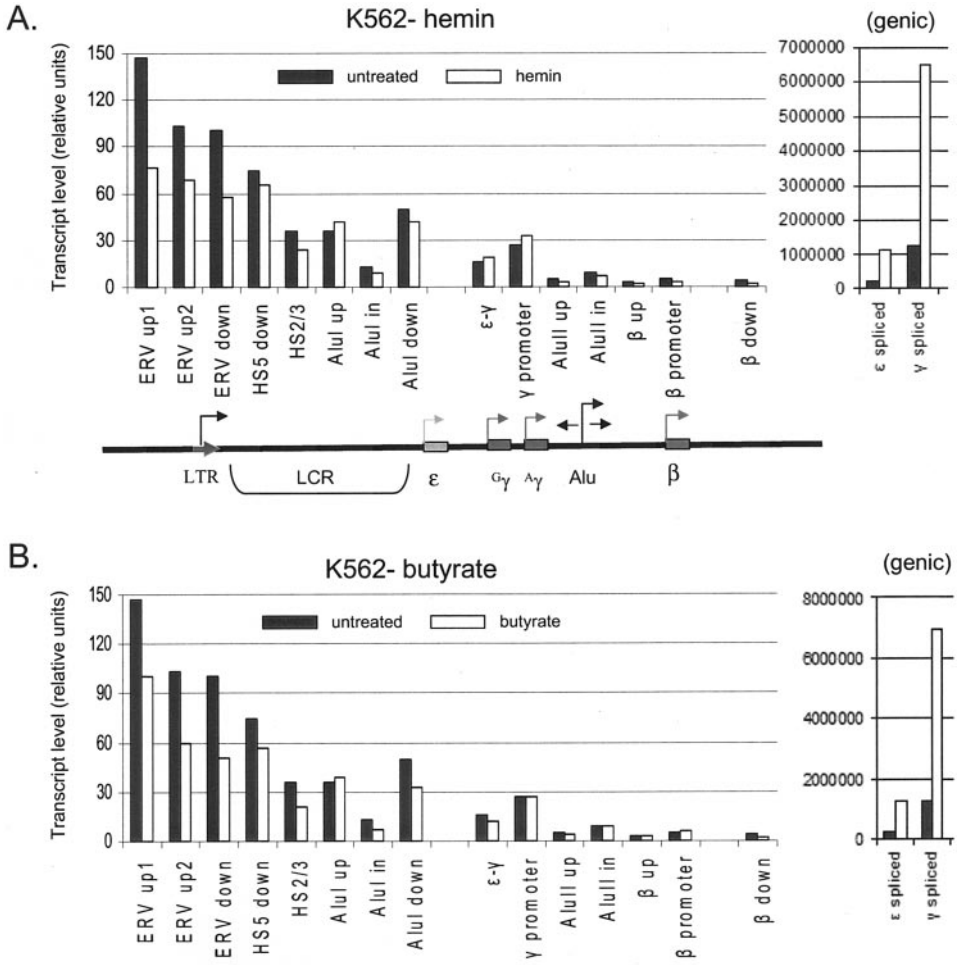

FIG. 2. K562 β-globin cluster transcript profile following hemin (A) and butyrate (B) treatment (real-time RT-PCR). Due to significantly higher spliced ε- and γ-globin steady-state levels, these values are juxtaposed in separate graphs to the right. Only the sense strand profile is shown for clarity.

Specific upregulation of induced intergenic and unspliced genic transcripts. The effect of Dicer knockdown on TSA-induced globin genic transcripts is particularly illuminating. Similar to intergenic transcripts, TSA-induced unspliced transcripts were strongly upregulated in Dicer knockdown cells, whereas spliced globin transcripts were much less affected (Fig. 4D and 5). This is demonstrated for splicing of both intron 1 and intron 2 of all globin genes analyzed (ε-, γ-, and β-globin) (Fig. 5). The analysis of the short intron 1 is especially illustrative, as both spliced and unspliced isoforms were simultaneously amplified by primers situated in exons 1 and 2 (Fig. 5B). This allowed for the observation of the distinct response of spliced and unspliced isoforms to Dicer knockdown in the same PCR (RNase protection analysis was not sensitive enough to pick up these transcripts). The specificity of the Dicer knockdown-dependent upregulation of TSA-induced intergenic and unspliced genic transcripts is underlined by the fact that unspliced actin and tubulin transcripts in the same experimental samples were not affected, as were control spliced actin levels (Fig. 4D and Fig. 5A). Occasionally, there was a slight increase of spliced globin transcripts in Dicer knockdown cells (e.g., ε-globin) (Fig. 4D). This is may be due to increased histone acetylation in the β-globin locus in Dicer knockdown cells, thus compounding the effect of TSA (see Fig. 7A).

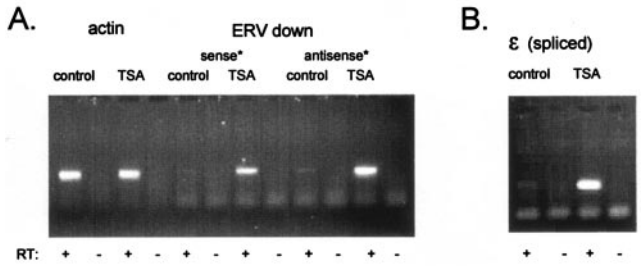

FIG. 3. TSA induction of HeLa cells. (A) Both sense and antisense intergenic transcripts are induced as illustrated for "ERV down" located just upstream of the LCR. As the emphasis was on induction efficiency, "ERV down" sense and antisense intergenic transcripts are shown at different cycle numbers. (B) TSA also induces genic transcription as illustrated by spliced ε-globin transcripts. For actin normalization, see panel A.

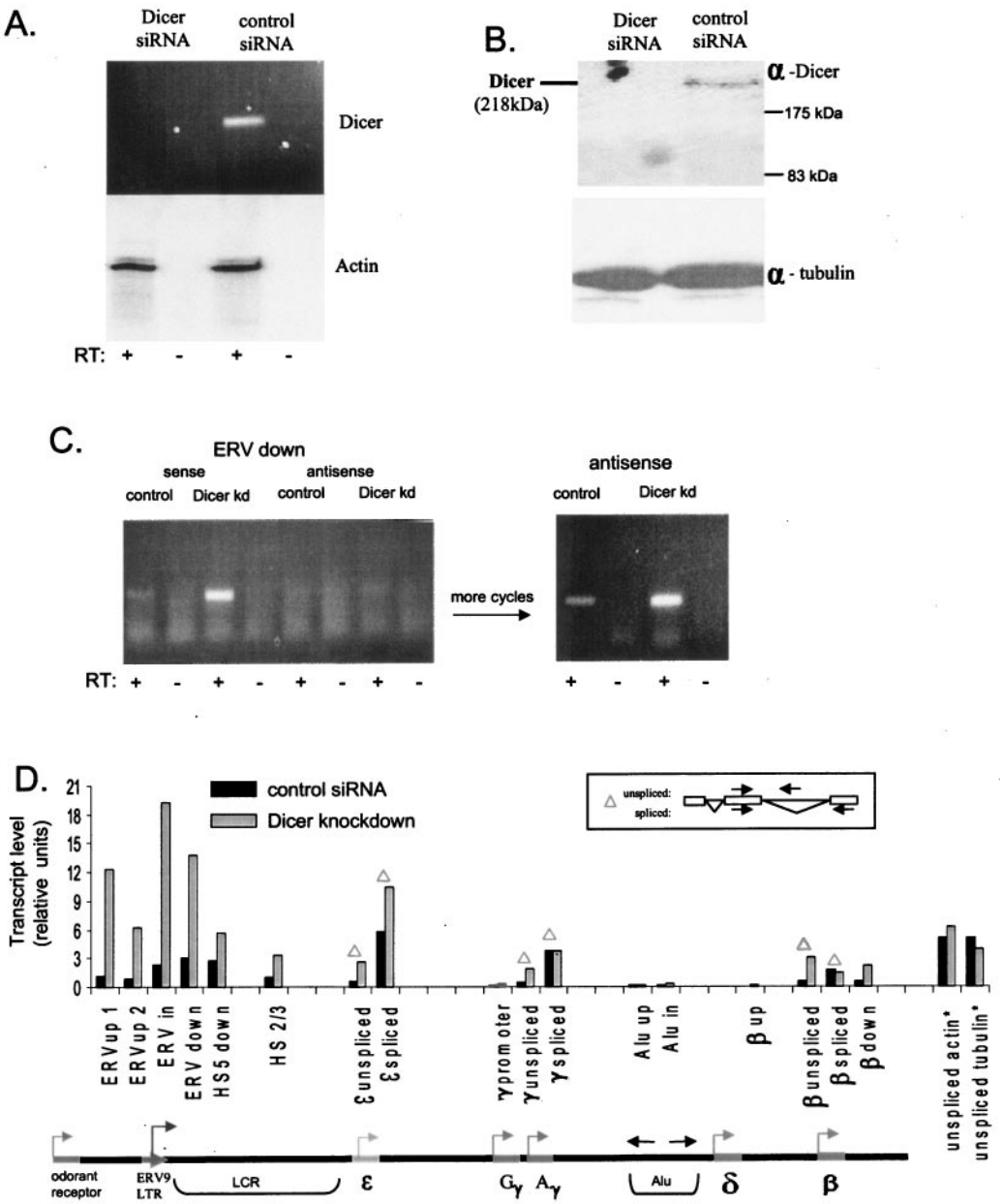

FIG. 4. Dicer knockdown considerably increases TSA-induced β-globin cluster intergenic and unspliced genic transcript levels in HeLa cells. (A) Dicer knockdown (RT-PCR analysis). (B) Dicer knockdown (Western analysis). (C) Effect of Dicer knockdown on the level of sense and antisense intergenic transcripts at "ERV down." Appropriate actin controls are shown in panel A. (D) Dicer knockdown effect on TSA-induced transcript levels in the β-globin gene cluster (real-time RT-PCR). A star indicates that unspliced actin and tubulin levels are not comparable with those in the β-globin gene cluster. Only sense transcripts are shown for clarity; △ marks genic elements; respective primer designs to detect spliced and unspliced isoforms are indicated in the inset. Only the spliced isoform is amplified under conditions used with primers separated by the ~850-bp intron 2 (see also Fig. 5). Suffixes "up" and "down" indicate positions 5′ and 3′, respectively, to the named element.

RNA fractionation experiments finally showed that the intergenic transcripts were still restricted to the nuclear RNA fraction following their knockdown-dependent increase, indicating that the upregulation occurred in the same compartment (Table 1). These findings add to the growing list of nuclear roles for Dicer and RNAi-related processes.

Nuclear RNAi-related processes. In the course of our RNA fractionation analyses, we discovered that Dicer RNA was reduced not only in the cytoplasmic but also in the nuclear fraction following Dicer siRNA treatment (Fig. 6D). This finding was unexpected, since the previous observation that siRNAs against introns did not elicit transcript downregulation argued that dsRNA-triggered RNAi is a strictly cytoplasmic process (13). Furthermore, a plasmid-derived human immunodeficiency virus transcript was only downregulated in the cytoplasmic but not the nuclear fraction in human cells (48). We therefore extended the RNA fractionation studies to confirm that RNAi can indeed reduce targeted RNA in the nuclear fraction.

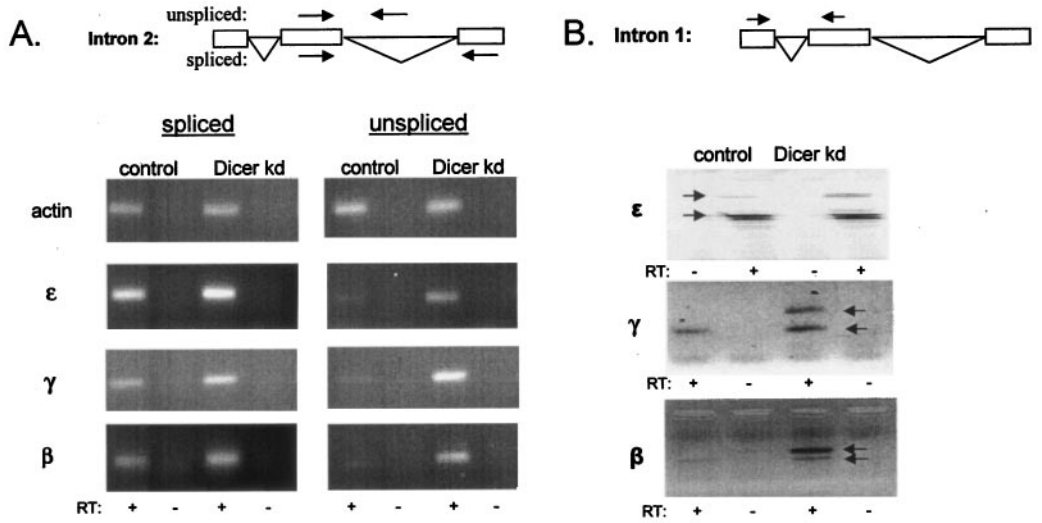

FIG. 5. Upregulation of β-globin gene cluster transcripts is specific for TSA-induced unspliced genic and intergenic transcripts. (A) Effect of Dicer knockdown (kd) on actin and β-like globin intron 2 spliced/unspliced transcript levels. (B) Effect of Dicer knockdown on β-like globin intron 1 spliced/unspliced transcript levels. The upper and bottom arrows indicate unspliced and spliced transcripts, respectively.

A reporter plasmid containing the β-globin gene under the control of a CMV promoter was modified to include a recognition site for an siRNA targeting endogenous PTB, a splicing factor (7). PTB was chosen since, like Dicer, it is not predicted to be targeted to the endoplasmic reticulum (ER) for translation (data not shown). This is important since fractionation analysis of ER-targeted mRNA may be confounded by the fact that the ER is contiguous with the outer nuclear membrane. Cotransfection of such a plasmid with PTB siRNA into HeLa cells should therefore allow us to simultaneously monitor siRNA targeting of both endogenously expressed and plasmid-derived transcripts in the same nuclear and cytoplasmic prep-

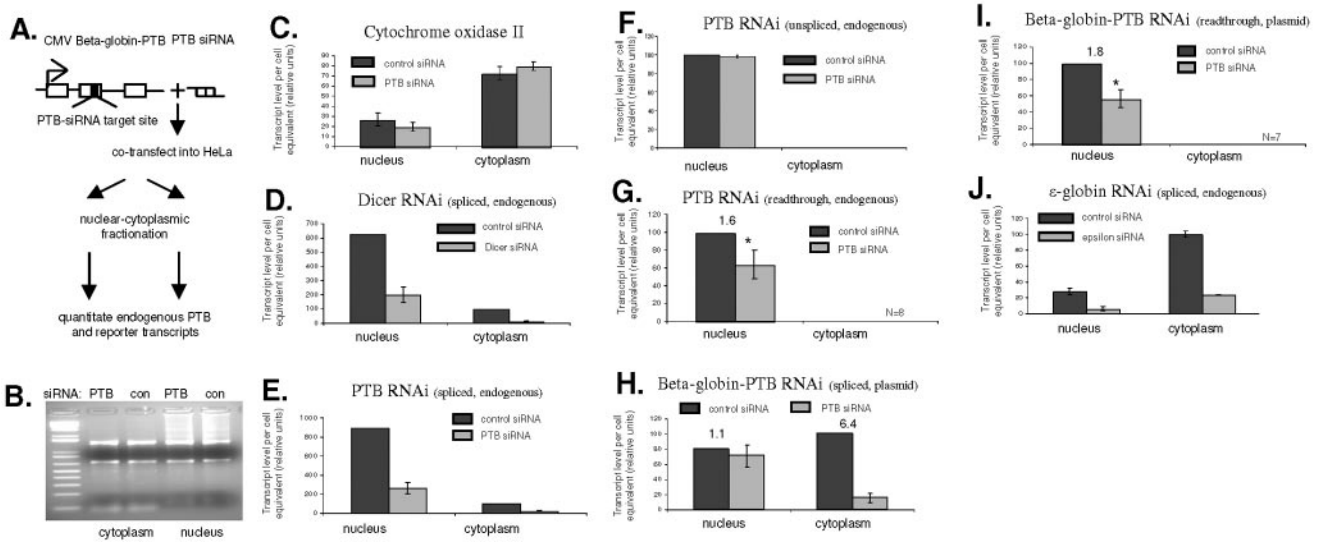

FIG. 6. Nuclear RNAi. (A) Diagram showing β-globin reporter construct with inserted PTB siRNA target sequence as well as experimental scheme for PTB siRNA-mediated knockdown experiments and analysis. (B) Fractionation (ethidium bromide-stained agarose gel) of nuclear and cytoplasmic RNAs following siRNA treatment. About five times more nuclear than cytoplasmic cell equivalents are shown. DNA size markers (values in nucleotides) were loaded in the first well. con, control. (C) Mitochondrially encoded cytochrome oxidase II message is largely recovered in the cytoplasmic fraction. (D) Dicer siRNA treatment effect on nuclear and cytoplasmic Dicer transcript levels (spliced). (E) Spliced PTB is equally knocked down in both fractions. (F) Unspliced PTB transcript levels are unchanged following PTB siRNA treatment. (G) A smaller but highly significant PTB siRNA-dependent decrease of PTB readthrough transcripts is observed in the same fractions. (H) Analysis of the same fractions as shown in panel B and employed in panels E to H shows that a plasmid-derived spliced message is only knocked down in the cytoplasmic fraction. (I) Readthrough transcripts derived from the same plasmid are reduced in an siRNA-dependent manner. (J) ε-Globin siRNA treatment causes ε-globin transcript knockdown in both nuclear and cytoplasmic fractions. (C to J) Real-time RT-PCR analyses. Values on top of the bars indicate severalfold knockdown of analyzed RNAs; RNA was harvested 16 h after siRNA transfection. (B to I) HeLa. (J) K562. $^*P < 0.001$ (Student's t test, paired).

arations (Fig. 6A). The integrity and specificity of the RNA fractions were also confirmed and are illustrated in Fig. 6B and C. As was already indicated by the strictly nuclear localization of unspliced and intergenic transcripts (Table 1), the cytoplasmic fraction is virtually free from nuclear RNA, while ~20% of the cytoplasmic RNA was carried over into the nuclear fraction as inferred from the cytoplasmic marker transcript cytochrome oxidase II, a mitochondrial transcript (Fig. 6C). This level of cross-contamination cannot quantitatively account for the above-described results or the following results.

As with our findings on knockdown of nuclear Dicer RNA, PTB transcripts are significantly depleted in both the nuclear and cytoplasmic fractions following siRNA treatment (Fig. 6E). Interestingly, nuclear-restricted readthrough RNA from downstream of the poly(A) site was downregulated by siRNA treatment (Fig. 6G), albeit to a lesser extent than spliced mRNA. In contrast and consistent with previous reports, however, unspliced transcripts were not affected (Fig. 6F). Unlike endogenous PTB, plasmid-derived β-globin mRNA is only depleted in the cytoplasmic fraction (Fig. 6H); yet again, there is significant siRNA-dependent reduction of nucleus-restricted transcripts from downstream of the predicted poly(A) site (Fig. 6I, "readthrough"). To extend these studies, we then targeted the endogenously expressed ε-globin mRNA in K562 cells. Consistent with the hypothesis that the type of template from which a transcript was generated plays an important role in determining the accessibility of an siRNA to their target, ε-globin message was considerably reduced in both fractions (Fig. 6J).

From the above-described analysis, we conclude that RNAi (-related processes) can function in the nucleus of human cells. Potential differences in RNA processing kinetics, localization, and the relative ratio of siRNA to target RNA between the endogenous and plasmid-derived genes studied may determine the accessibility of a transcript to RNAi. Moreover, RNAi-related processes in this compartment may be closely coupled to transcription, as indicated by the effects of siRNA treatment on readthrough transcript levels. Since the siRNA was targeted against the exons of the gene (Fig. 6A) but readthrough transcripts and not unspliced transcripts were downregulated, siRNA targeting may occur after splicing but before polyadenylation. Alternatively, transcripts that are spliced and yet fail to undergo polyadenylation may be accessible to siRNAs. In any case, these results suggest that RNAi-related processes are closely coupled to transcription and occur in the nucleus.

In support of these findings, it has now been reported that siRNA-dependent RNAi may indeed operate in the nucleus and may knock down nucleus-restricted transcripts (28, 37, 40). The present study therefore extends nuclear RNAi for "normal" mRNAs. Overall, our data make a strong case for nuclear RNAi-like activities.

Dicer knockdown is linked to chromatin activation. Since many of the previously described nuclear RNAi-related processes have been linked to the formation of silent chromatin (33, 38, 45), including vertebrate centromeres (15, 25, 27, 31), we investigated whether Dicer knockdown affects chromatin structure in HeLa cells at the noncentromeric β-globin locus. We initially assessed histone H4 acetylation before, during, and following treatment with TSA (Fig. 7A). As expected, TSA increased histone acetylation significantly, demonstrating that histone deacetylation, and therefore chromatin silencing of the β-globin gene cluster, is indeed an ongoing process in nonerythroid cells.

Importantly, histone H4 acetylation was consistently and robustly increased at each stage in Dicer knockdown cells. This is illustrated for "ERV down" (Fig. 7A) and was also observed at other elements in the β-globin locus (data not shown). The high relative enrichment in H4 hyperacetylation in Dicer knockdown cells following withdrawal of TSA is consistent with the observation in *Schizosaccharomyces pombe* that RNAi is particularly but not exclusively required for the (re-) establishment of heterochromatin outside centromeres subsequent to TSA treatment (19, 24, 38). The increase in H4 acetylation in the absence of TSA (Fig. 7A and B), however, suggested an additional role for RNAi in the maintenance of silent chromatin (15, 19, 24, 25, 33, 45).

We consequently studied the nature of potential coordinate changes in histone tail modifications in otherwise-untreated Dicer knockdown cells (Fig. 7B to D). The accumulation of acetylated H4 following Dicer knockdown appears to be broadly distributed (Fig. 7B), in agreement with this modification marking chromatin domains in the β-globin gene cluster rather than being targeted to particular genes and control elements (39). In striking contrast to the acetylation of histone H4, acetylation of histone H3 lysine 9 was not affected by Dicer knockdown (Fig. 7C). We note that the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) locus showed reduced H3K9 acetylation following Dicer knockdown (about 25% of control). This is possibly a consequence of the known reduced growth rate of Dicer knockdown cells and the predicted suppression of glycolysis under such conditions. Levels of H3K4 dimethylation, another histone modification characteristic of active chromatin which, like H4 acetylation, had been shown to mark whole chromatin domains such as that in the human Hox gene cluster (4), were also studied. Similar to H4 acetylation, Dicer knockdown generally promoted H3K4 dimethylation (Fig. 7D). We also investigated markers indicative of repressed chromatin (HP1α, HP1γ, and H3K9/27 trimethylation), but only very little chromatin was precipitated, suggesting either that these modifications may be of less importance in the regulation of the β-globin locus or that they failed to be effectively recognized by the antibodies (data not shown).

Taken together, our results show that chromatin silencing of the β-globin locus of nonerythroid cells is an ongoing, dynamic process which may require the RNAi machinery as indicated by the Dicer knockdown-dependent chromatin activation.

DISCUSSION

These studies began by the testing of chromatin activation-related models for the function of intergenic transcription. We compared intergenic transcript abundance with histone H4 acetylation either in the context of embryonic-fetal ε/γ-globin (K562) or adult β-globin (Fibach culture) expression. However, our results are inconsistent with such models. Importantly, whereas K562 intergenic transcript levels are elevated upstream of the ERV element in K562 cells, H4 acetylation in the same region is considerably weakened. Further notable discrepancies between intergenic transcript abundance and histone acetylation are observed in the adult β-globin domain of

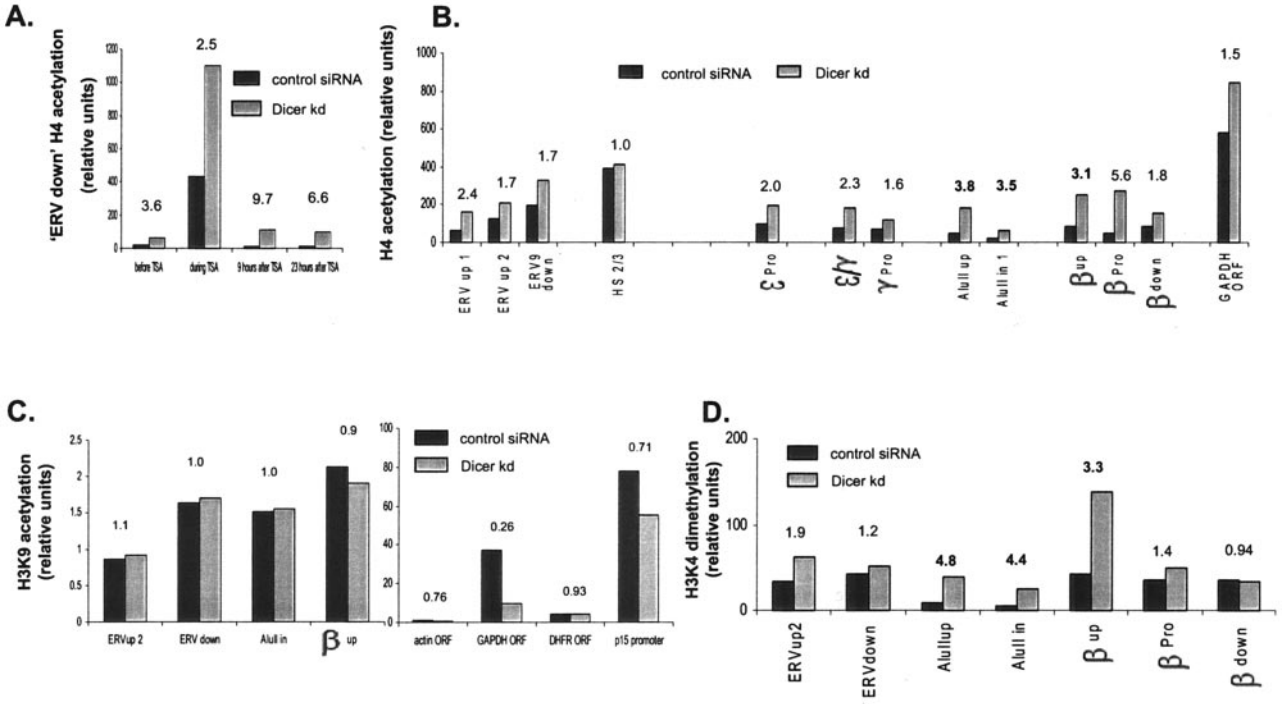

FIG. 7. Chromatin activation of the β-globin gene cluster after Dicer knockdown in HeLa cells (ChIP analysis by real-time PCR). (A) Time course analysis for "ERV down" shows increased histone H4 acetylation in Dicer knockdown (kd) cells before, during, and especially after treatment with TSA. (B) Widespread increase in histone H4 acetylation in Dicer knockdown cells in the absence of TSA. ORF, open reading frame. (C) Histone H3K9 acetylation remains unchanged in Dicer knockdown cells except for a decrease for GAPDH. DHFR, dihydrofolate reductase. (D) Histone H3K4 dimethylation is generally increased in Dicer knockdown cells. Values on the top of bars indicate the ratio of real-time PCR signal for Dicer knockdown to control siRNA-treated cells.

K562 and around the γ-globin genes in adult erythrocytes. To further test the possibility that intergenic transcription correlates with genic transcription, we treated K562 with transcriptional activators of globin gene expression, hemin and butyrate. As expected, genic transcription strongly increased; however, intergenic transcripts were unaffected, further arguing against activation-linked models of intergenic transcription.

As a number of noncoding transcripts had been reported to be turned over by RNAi, typically linked to chromatin silencing, we decided to investigate an involvement of RNAi in the regulation of the human β-globin gene cluster. This was also supported by the detection of both sense and antisense intergenic transcripts. Strikingly, we find that induced β-globin intergenic transcripts were specifically upregulated in Dicer knockdown cells. Induced unspliced globin transcripts were also upregulated, which may be explained by transcriptional readthrough of intergenic transcription into the globin genes (data not shown). It appears that the intergenic transcription process itself is not accessed by typical mRNA processing but instead by an RNAi-related turnover mechanism. We note that although we robustly detected RNA polymerase II over the active globin genes in erythroid cells by ChIP, almost no signal above background was obtained for the intergenic regions (data not shown). Interestingly, a new type of transcription has recently been identified in plants and was found to relate to RNAi-mediated chromatin silencing (20, 32).

Furthermore, chromatin silencing in the β-globin locus appears to be a highly dynamic process in nonerythroid cells, and consistent with an involvement of RNAi in this process, Dicer knockdown correlated with a shift towards a more activated chromatin state. We speculate that such Dicer-dependent chromatin silencing is mediated by intergenic transcription itself, although the chromatin activation, unlike the intergenic transcript effect in Dicer knockdown cells, is not exclusively restricted to the β-globin locus. This may relate to observations in *Drosophila melanogaster* and *Arabidopsis thaliana*, in which the inhibition of RNAi-related proteins resulted in genome-wide changes in chromatin structure, illustrating the importance of RNAi-related processes in regulation of the global chromatin state (32, 33). Elements such as the ERV solo LTR upstream of the LCR may serve as nucleation centers for such chromatin regulation. Notably, genes in proximity to solitary LTRs, akin to the one found upstream of the human β-globin LCR, are both posttranscriptionally and transcriptionally silenced in an RNAi-dependent manner during the mitotic growth of fission yeast (38). It would be interesting to examine whether such global regulation was related to the pervasive, genome-wide intergenic transcription that occurs in all organisms investigated towards this effect (5, 26, 41, 42).

In erythroid cells, rather than silencing the whole β-globin cluster, intergenic transcription may help to silence chromatin by default in regions not bound by activatory transcriptional regulators. Hence, a loss of such transcription factors in the embryonic-fetal domain during adult erythropoiesis, or their absence in the LCR in nonerythroid cell lineages, may allow

for intergenic transcription to effectively silence chromatin in these domains.

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