Chromatin, Gene, and RNA Regulation

Molecular Cancer Research

Dual Promoter Usage as Regulatory Mechanism of let-7c Expression in Leukemic and Solid Tumors

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Abstract

Let-7c, an intronic microRNA (miRNA) embedded in the long non-coding gene *LINC00478*, can act as a tumor suppressor by targeting oncogenes. Previous studies indicated that in acute promyelocytic leukemia (APL), a subtype of acute myelogenous leukemia (AML) bearing the leukemia promoting PML/RARα fusion protein, let-7c expression seems to be controlled by the host gene promoter, in which canonical Retinoic Acid Responsive Elements (RAREs) are bound by PML/RARα in an all transretinoic acid (ATRA)–sensitive manner. Here, let-7c transcriptional regulation was further investigated and a novel intronic promoter upstream of the pre-miRNA was identified. This new promoter has transcriptional activity strongly indicating that at least two promoters need to be considered for let-7c transcription: the distal host gene and the proximal intronic promoter. Therefore, epigenetic modifying enzymes and histone acetylation and methylation status were analyzed on both let-7c promoters. It was demonstrated that ATRA treatment leads to let-7c upregulation inducing a more open chromatin conformation of the host gene promoter, with an enrichment of epigenetic marks that correlate with a more active transcriptional state. Conversely, the epigenetic marks on the intronic promoter are not significantly affected by ATRA treatment. Interestingly, in solid tumors such as prostate and lung adenocarcinoma it was found that both host and intronic promoters are functional. These data suggest that while the host gene promoter may control let-7c expression in AML, in a nonleukemic tumor context instead the intronic promoter contributes or preferentially regulates let-7c transcription.

Implications: Alternative promoter usage represents a regulatory mechanism of let-7c expression in different tissues.
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Introduction

microRNAs (miRNA) are small noncoding RNAs that regulate gene expression post-transcriptionally (1). Their function has been linked to the regulation of several cellular processes, including the effects of oncogenes or tumor-suppressor genes in neoplastic transformation (2). Current evidence suggests that a significant portion of the human genome is regulated by miRNAs, and many reports have demonstrated that miRNAs expression is deregulated in human development and disease, including that of the hematopoietic system (1, 2). Although the biologic importance of miRNAs is becoming clearer, the regulation of their expression is not fully understood (3). Therefore, the identification of genetic and epigenetic elements responsible for transcriptional regulation of miRNAs transcription may aid in the understanding of pathways governing the biology of miRNAs.

Let-7c, a member of the let-7 family first discovered in *Caenorhabditis elegans* (4), is highly conserved across the animal phylogeny from *C. elegans* to human (5) providing evidence for the role of miRNAs as essential regulators of gene expression in various organisms. The human let-7 family contains 13 members located on nine different chromosomes, and is widely viewed as a family of tumor-suppressor miRNAs (6). Consistent with this activity, the expression of several let-7 family members is downregulated in many cancer types when compared with normal tissue and

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during tumor progression (7–12). Previously, we found that in acute promyelocytic leukemia (APL; 13), a subtype of acute myelogenous leukemia (AML) bearing the leukemia-promoting PML/RARα fusion protein, let-7c expression was downregulated in APL blasts at diagnosis, compared with *in vitro*–differentiated normal promyelocytes (11). Moreover, in AMLs, expression of the let-7c miRNA was found downregulated in patients with t(8;21) and inv(16; ref. 9). In contrast, let-7c overexpression impaired cellular proliferation and tumor development in xenografts and in autochthonous models of non–small cell lung cancer (14). Several reports have linked let-7 miRNAs to human cancer through their negative regulation of post-transcriptional expression of multiple oncogenes, including *RAS*, *MYC*, and *HMGA2*, as well as cell-cycle progression genes (8, 15–19). Recently, we also found that PBX2, a protein involved in leukemiogenesis, is a novel target of let-7c and that a cross-talk between let-7c and PBX2 may contribute to the AML phenotype (20). We also showed that ectopic expression of let-7c promotes granulocytic differentiation of AML cell lines and primary blasts (20), suggesting that perturbation of let-7c expression could have therapeutic value in AML.

The let-7c miRNA is located in the sixth intron of the host gene *LINC00478* and is organized in a cluster with miR-99a and miR-125b-2 on chromosome 21 (ref. 21). Until recently, it was generally accepted that intronic miRNAs are transcribed as result of their host gene transcription (22–25), and we also observed a coordinated regulation of let-7c expression with its host gene, suggesting that its transcription might be controlled by the host gene promoter. Interestingly, we identified canonical RARE elements bound by PML/RARα in the let-7c host gene promoter, and we found that this binding is released in response to all transretinoic acid (ATRA; ref. 11). However, the concept is emerging that processing from the host gene primary transcript is not the sole pathway for intronic miRNA generation (26). About let-7c, recent studies identified different putative intronic transcriptional start sites (TSS; refs. 27–31), suggesting that its promoter has yet to be defined.

In the present study, we mapped a novel TSS, consistent with the existence of an intronic promoter region and strongly supporting the hypothesis that at least two promoters have to be considered as functional in let-7c transcription: the distal host gene and the proximal intronic promoter. Of note, we provide evidence that the host gene promoter is responsive to ATRA and essentially the only one active in AML cells, whereas in solid tumors also the intronic promoter is regulating let-7c expression as well. These data led us to propose a model in which usage of either promoter could represent a regulatory mechanism of let-7c expression in different tissues. Thus, let-7c could provide a useful resource for the study of both normal development and pathogenesis.

Materials and Methods

Reagents and antibodies

Antibodies and reagents were purchased from the following companies: anti-PML (PG-M3 and H-238), anti-RXR (ΔN 197), and anti-p300 (N-15; Santa Cruz Biotechnology); anti-HDAC1 (3284; Sigma-Aldrich); anti-H3K9me3 (ab8898; Abcam); anti-H3K4me2 (07-030), anti-H3K4me3 (07-473), anti-acetyl H3 PAN (06-599), anti-acetyl Histone H4 (06-598), anti-H3K9ac (07-352), anti-H3K14ac (07-353), and anti-H3K27ac (07-360; Millipore). ATRA was from Sigma-Aldrich.

Cell cultures

Human leukemic cell lines NB4, HEL, HL-60, KG1, K562, SKNO-1, THP-1, and UF-1 were maintained in RPMI (Life Technologies Invitrogen) with 10% (v/v) fetal calf serum (FCS; Hyclone, Perbio), penicillin (200 U/mL), and streptomycin (100 mg/mL). For cell lines BV173, GDM-1, KG-1a, KASUMI-1, and PL-21 FCS was 20% (v/v). OCI-AML3 cells were maintained in α-Minimum Essential Medium (MEM; Life Technologies Invitrogen) 20% (v/v) FCS, penicillin, and streptomycin.

Human carcinoma cell lines H1299, A549, MCF7, and A2780 were maintained in RPMI (Life Technologies Invitrogen) with 10% (v/v) FCS (Cambrex Bio-Science, Verviers), penicillin (200 U/mL), and streptomycin (100 mg/mL). SKBR3, SH-SY5Y, HepG2, and HCT-116 cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies Invitrogen) supplemented with 10% FCS or 15% FCS for SH-SY5Y. LNCaP, were grown in RPMI supplemented with 10% FBS, penicillin, glutamine and 4.5 g/L glucose. DU145 and PC3 were grown in DMEM with 10% FBS, penicillin, and glutamine. Immortalized prostate cancer cells, C17IM and C27IM, were cultured as described previously (32).

5′-rapid amplification of cDNA ends

The 5′ end of the primary transcripts of let-7c was determined using the 5′RACE System for Rapid Amplification of cDNA Ends according to the manufacturer's instruction (Invitrogen).

First strand cDNA was synthesized from 5 μg of DNase-treated RNA, prepared from LNCaP cells, using a gene-specific antisense oligonucleotide (GSP1, 5′-ACGTAAAATGTCTTCTGAC-3′). After first strand cDNA synthesis, the mRNA template was removed by treatment with the RNase Mix. Unincorporated dNTPs, GSP1, and proteins were separated from cDNA using an S.N.A.P. column. A homopolymeric tail was then added to the 3′-end of the cDNA using TdT (terminal deoxynucleotidyl transferase) and dCTP. Tailed cDNA was then amplified by PCR using a gene-specific primer (GSP2, 5′-AGGGTTTGGACCAGGATCT-3′), which anneals 3′ to GSP1, and an abridged anchor primer (AAP) specific for the oligodC tail that permits amplification from the homopolymeric tail. Subsequently, a nested PCR was performed using a GSP3 primer (5′-CAAAATGCTATACACAGTGCCG-3′), which anneals 3′ to GSP2, and an Abridged Universal Amplification Primer, which anneals with the AAP. PCR products were cloned into the TA cloning vector pCR2.1 (Invitrogen) and sequenced.

Plasmid constructions, transfections, and luciferase assays

DNA fragments upstream of the pre–let-7c sequence (nt −2,195 to nt −498) and of the first exon of *LINC00478* sequence (nt −1,960 to nt 1) were PCR amplified from human placenta genomic DNA using specific primers (Supplementary Table S1) and inserted in the pGL3 basic reporter vector (Promega) upstream of the luciferase gene. Deletion mutants were generated by restriction site digestions and standard cloning techniques. Briefly, Δ#1-pGL3-prom-700, Δ#2-pGL3-prom-200, and Δ#3-prom-90 were generated by digesting construct pGL3 intronic let-7c prom (1,700 bp) with SacI/BglII (nt −2,195/−1,224) and SacI/StuI (−2,195/−878) or SacI/PspOMI (−2,195/−757), respectively. After digestion, to produce blunt-ended fragments, Klenow fragment of *E. coli* DNA polymerase I (New England BioLabs, Inc.) was used. All plasmids were verified by sequencing. A total of 2×10^6 NB4 cells were transiently transfected, by electroporation (Nucleofector 4D system; Lonza; buffer SF), with 500 ng of firefly luciferase reporter plasmid containing either the let-7c intronic or the host gene promoter. The pGL3 empty vector was used as negative control. Of note, 10 ng *pRL-TK* vector providing constitutive expression of Renilla luciferase was cotransfected as an internal control to correct differences in both transfection and harvest efficiencies and no consistent changes in raw Renilla luciferase were observed. After 5 hours from transfection, the cells were treated with ATRA (1 μmol/L) and 24 hr post-treatment were lysed and luciferase activity was quantified using the Dual Luciferase Reporter Kit (Promega Inc.) according to the manufacturer's instructions.

LNCaP and H1299 cells were seeded in 60-mm cell culture dishes and the day after transiently cotransfected by Lipofectamine 2000 (Life Technologies), with 10 ng of Renilla luciferase *pRLTK* and 2 μg of pGL3 intronic let-7c or host gene let-7 promoter or the deletion mutants (Δ#1-pGL3-p700, Δ#2-pGL3-prom-200, and Δ#3-prom-90). Cells were harvested 24 hours post-transfection, lysed and luciferase activity quantified as above. Firefly luciferase activity of each sample was normalized to Renilla luciferase activity. Results are expressed as fold activation relative to the basal activity of pGL3 basic empty vector.

Molecular studies

Total RNA was isolated using TRizol Reagent (Invitrogen). Quantification of *LINC00478* host gene expression and of pri-miR-let-7c, −99a and −125b (the primers used are listed in Supplementary Table S1) was performed using SYBR Green-based qPCR as described previously (11). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene expression was used as endogenous control. Analysis of mature miRNA was performed using the TaqMan method according to the manufacturer's protocol (Applied Biosystems). RNU19 served as endogenous control.

Northern blot analysis of larger RNAs (pri-miR-let-7c and pri-miR-99a) were performed by separating 30 μg of total RNA in 1.2% agarose gels under denaturing conditions. RNA marker (Ambion Millenium RNA Markers, Life Technologies) was used.

RNA was transferred and UV cross-linked to nylon membranes (Zeta-Probe GT; Bio-Rad), which were then hybridized to specific DNA probes. For the generation of the Northern probes a 237 bp DNA fragment corresponding to nucleotides +182 to +419 respect to the intronic TSS for let-7c probe *or* a 257 bp DNA fragment from nucleotides −305 to −48 for pri-miR-99a probe was PCR amplified using specific primers indicated in Supplementary Table S1. Radioactive labeling of the probe was performed using the Random Primer Labeling Kit (Invitrogen). Hybridization was done overnight at 42°C.

Chromatin Immunoprecipitation Assay

Cross-linked chromatin from NB4, Hep-G2, SKBR3, LNCaP, C17IM, or C27IM cells was immunoprecipitated with the indicated antibodies as described previously (11, 32). The genomic regions in the host gene and intronic promoter, close to the RARE sites or to TSS, were amplified with primers designed by the Primer Express software (Applied Biosystem) and indicated in Supplementary Fig. S1 and Supplementary Table S1. Quantization of immunoprecipitated DNA was performed in triplicate on an Applied Biosystem 7500 Real-Time PCR System SDS v1,2, using the SYBR green dye detection method (11). ChIP (chromatin immunoprecipitation) assay results are evaluated by the $\Delta\Delta C_t$ method. Values of each immunoprecipitated sample are expressed as percentage relative to their respective input and by subtracting the values obtained in the negative controls (no antibody).

Bioinformatics

Evolutionary sequence conservation was examined using the UCSC Genome Browser track (http://genome.ucsc.edu/). Sequence alignment of genomic DNA sequences was performed using NCBI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST; algorithm: blastn) and software from Genetics Computer Group (GCG; Campbell, CA). Identification of transcription factor–binding sites was performed using Genomatix suite programs MatInspector (http://www.genomatix.de), TESS (http://www.cbil.upenn.edu/cgi-bin/tess), and Transfac matrix Database (v.7.0) track on UCSC Genome Browser.

Statistical analysis

All results are expressed as mean ± SEM of at least three independent experiments. All quantitative real-time PCR (q-PCR) experiments were carried out in triplicate. Correlation analyses of host gene and let-7c expression were carried out in Excel. All analyses were two-tailed and were considered statistically significant when $P < 0.05$ (Student t test).

Results

Identification of a novel TSS for let-7c miRNA

Because several studies revealed different let-7c putative intronic TSS (27–30) the promoter of let-7c has yet to be fully

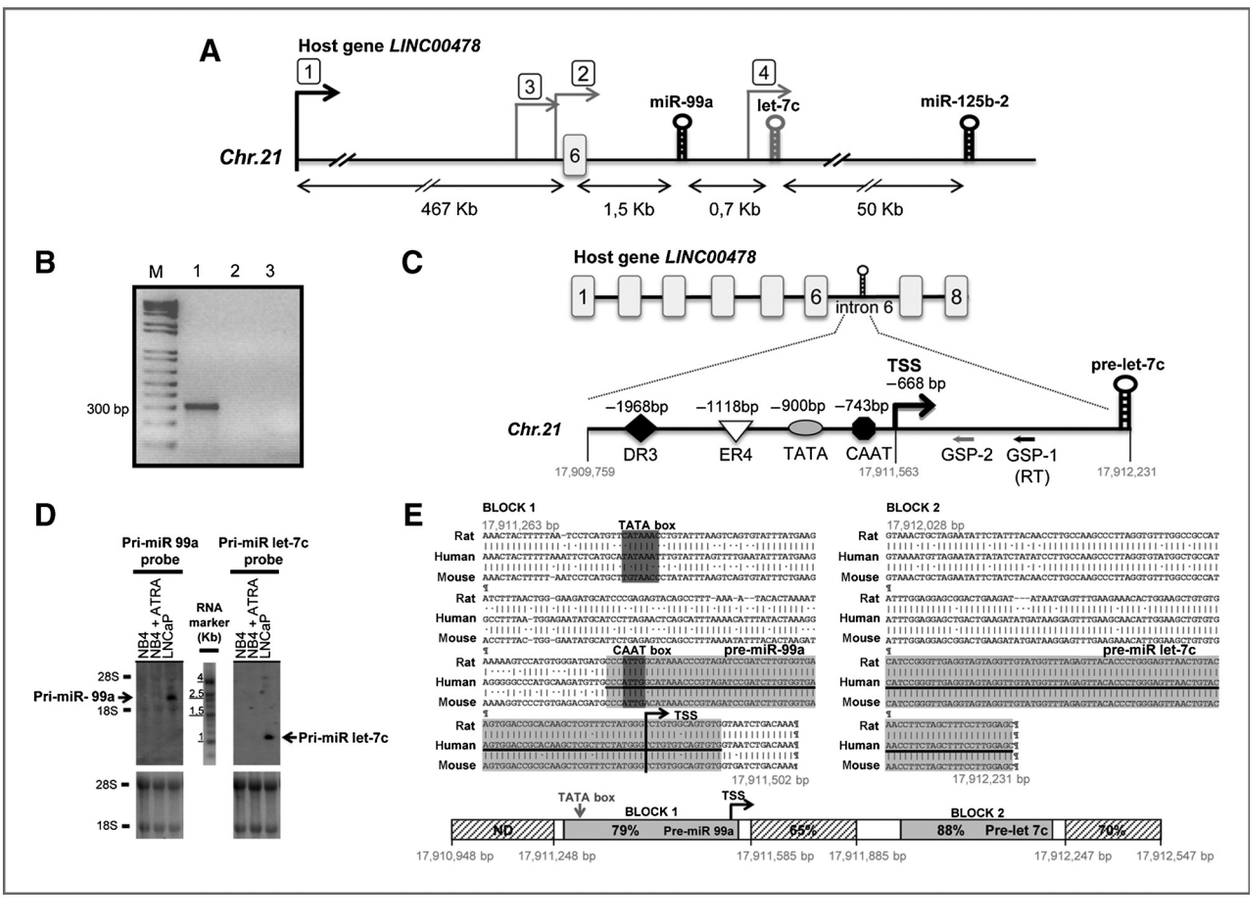

Figure 1. Identification of a novel let-7c TSS. A, genomic organization of the miR cluster containing let-7c. 1, TSS of the host gene LINC00478. 2, putative TSS position predicted by Ozsolak et al. (28); 3, Corcoran et al. (29); and 4, Monteys et al. (30). B, 5′RACE mapping of intronic let-7c gene TSS. Lane 1, cDNA from LNCaP poly-(dC) tailed and amplified by PCR using primers GSP-2 and GSP-3 for nested PCR and a universal primer (AAP) that anneals to the polyC tail. Lane 2, not tailed cDNA; lane 3, no template as control. M, Marker 1 Kb Plus DNA ladder (Life Technologies) is indicated. C, schematic representation of the novel region identified by 5′RACE, containing regulatory elements upstream of the pre–let-7c. Noncanonical RARE elements (DR3 and ER4) and conserved binding sites for transcription factors, such as TATA box or CAAT sequences, upstream of the let-7c initiation site, are indicated. Arrows outline the position of the primers GSP-1 and GSP-2 used in 5′RACE analysis. D, Northern blot analysis for the detection of miR-99a (left) and let-7c (right) primary transcript. Northern blot analysis of larger RNAs (pri-miR-let-7c and pri-miR-99a) was performed by loading total RNA in duplicate on a single gel to obtain twin filters hybridized with probes derived from nucleotides −305 to −48 (pri-miR-99a) or +576 to +652 (pri-miR-let-7c), respect to the novel intronic TSS identified. RNA marker is shown between the panels. Ethidium bromide staining of 28S and 18S ribosomal RNA (bottom) is shown as a loading control. Because the upregulation of let-7c after ATRA administration is described (11, 20), this parameter was used as positive control for the activity of ATRA (data not shown). E, alignments of let-7c upstream conserved regions named block 1 (assembly, GRCh37; Chr 21, 17,911,263–17,911,502) and block 2 (Chr21, 17,912,028–17,912,231) across the indicated species (top). The CAAT and TATA boxes and the positions of the pre–miR-99 and pre–miR-let-7c are also indicated. Schematic representation of conserved regions block 1 and 2 (bottom). The percentage of homology of the conserved blocks as well as outside the blocks is indicated. No percentage of homology was determined (ND) for the genomic region immediately upstream the block 1 (Chr 21, 17,910,948–17,911,248).

defined (Fig. 1A). To address this issue, we performed *in silico* analysis on the genomic region spanning 10 Kb upstream of the pre-miR sequence using ProScan v.1.7, BDGP and F/PROM. These bioinformatical tools were able to predict a single putative TSS, not yet described, located about 700 bp upstream of the pre–let-7c sequence, within the sixth intron of the let-7c host gene, *LINC00478*. This region presents regulatory elements consistent with a Pol II–associated promoter and –conserved binding sites for transcription factor such as CAAT and TATA boxes. To experimentally validate this putative TSS, we performed 5′-rapid amplification of cDNA ends (RACE) and obtained a single RACE product near the pre–let-7c sequence (Fig. 1B). After DNA sequencing of this product, we were able to localize the TSS at −668 from pre–let-7c, overlapping the TSS identified *in silico* (Fig. 1C). Because the novel TSS identified falls immediately downstream of pre–miR-99a sequence, to demonstrate that the termination event was not an artifact of the secondary structure created by the folding of the pre–miR-99a blocking the reverse transcription, we performed Northern blotting analysis using specific probes designed to detect pri-miRNA transcripts from the miR-99a or let-7c. To avoid cross hybridization with pri-miRNAs of other let-7 family members, the pri-miR-let-7c probe was complementary to unique sequences outside the pre-miR

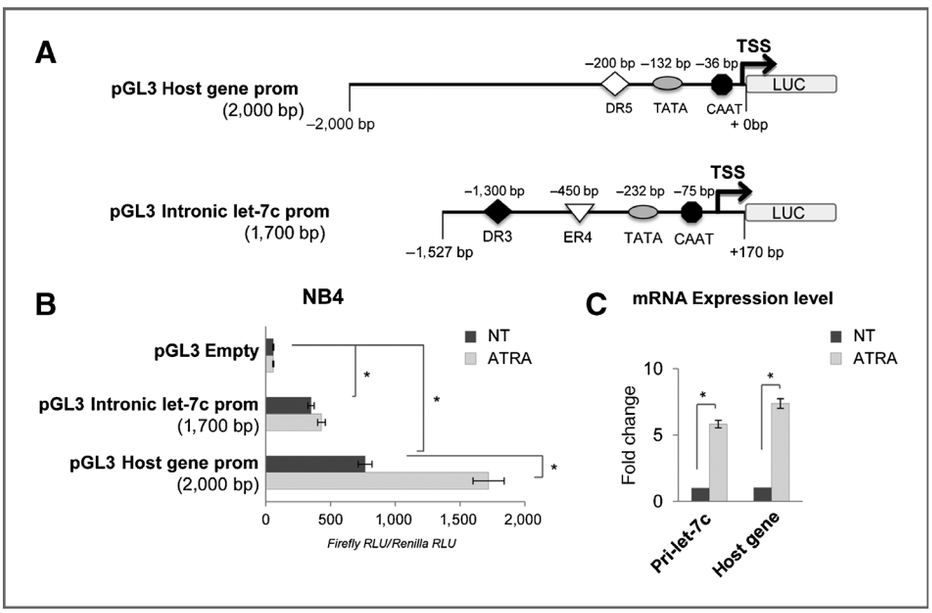

Figure 2. Transcriptional activity of let-7c intronic promoter in leukemic cells. A, schematic representation of the pGL3 intronic and host gene let-7c promoters plasmids used in transient transfection assays. The canonical and noncanonical RARE elements (DR5, DR3, and ER4) are indicated. B, human NB4 cells were transiently transfected with the indicated luciferase reporter vectors. The upstream region of the 5′UTR of the host gene cloned into a firefly luciferase reporter gene was used as positive control. Luciferase activity was assayed in untreated (NT) and ATRA-treated cells (1 μmol/L, 24 hours). For transfection efficiency values were normalized to the activity of a cotransfected renilla luciferase plasmid *pRLTK*, and expressed relative to the basal activity of pGL3 empty control vector. The normalized luciferase activity, as mean of at least three independent experiments done in duplicate, is shown. Error bars, SEM ($n = 3$). *, $P < 0.05$ (Student *t* test). C, RNA quantification, using SYBR Green-based real time quantitative Reverse Transcription PCR (qRT-PCR), of pri-let-7c and *LINC00478* host gene in untreated (NT) and ATRA-treated NB4 cells. GAPDH gene expression was used as endogenous control. Results (mean ± SEM of three independent experiments) are expressed as fold changes in ATRA-treated versus -untreated cells. *, $P < 0.05$ (Student *t* test).

sequences (Supplementary Fig. S1B and Supplementary Table S1). As shown in Fig. 1D, we found that pri-miR-99a and pri-let-7c probes detected two independent transcripts on LNCaP cells (approximately 2.7 Kb for pri-miR-99a; 1.1 Kb for pri-let-7c; Fig. 1D), thus, demonstrating the existence of a transcript containing let-7c but not miR-99a and *vice versa*. Interestingly, we did not detect any transcript in the leukemic cell line NB4 untreated or treated with ATRA (Fig. 1D), suggesting that the intronic promoter does not work in this cellular context.

In addition to the experimental identification of TSS, mapping of intergenic miRNA promoters is based on the phylogenetic conservation among species (33–35). Therefore, we compared the region upstream of the pre–miR-let-7c sequence among some vertebrate orthologs. We identified two highly conserved sequences, which we named block 1 and 2.

Block 1 spanning a region from −885 to −646 bp upstream from the pre–let-7c and partially overlapping pre–miR-99a sequence (from −70 to +10 bp respect to intronic TSS), is 300 bp long and 79% identical between mouse, rat, and human (Fig. 1E). This sequence includes important regulatory elements, such as TATA and CAAT boxes, located at the 3′ end of the conserved region, 232 and 75 bases upstream of the TSS, respectively. Block 2 is 294 bp long and is 88% identical between mouse and human. Moreover, the alignment of genomic regions located immediately outside the conserved blocks with mouse genome produced 65% of identity upstream (Chr 21: 17,911,585-17,911,885) and 70% downstream (Chr 21: 17,912,247-17,912,547) the block 2. Instead, the alignment of a genomic region immediately upstream the block 1 (Chr 21: 17,910,948-17,911,248) revealed no significant similarities with the mouse and rat genomes and, therefore, no percentage of homology could be established (Fig. 1E).

Transcriptional activity of let-7c intronic promoter in leukemic cells

To functionally characterize the novel intronic let-7c promoter, a fragment consisting of 1,700 bp upstream of the new TSS was cloned into a firefly luciferase reporter vector and transfected into NB4 cells (Fig. 2A). We have previously demonstrated that let-7c expression is increased in these cells upon ATRA-induced differentiation (11). Thus, we took advantage of this model to evaluate the ability of this DNA fragment to drive transcription and, if so, whether it was responsible for the ATRA-dependent increase of let-7c expression in these cells. As positive control, a fragment of 2,000 bp upstream of the 5′ untranslated region (UTR) of the host gene was fused to the luciferase gene (Fig. 2A). The luciferase assays demonstrated that the newly identified intronic regulatory region has transcriptional activity, indicating that it could work as a functional promoter. Nevertheless, in NB4 leukemic cells the host gene promoter elicited approximately 2-fold higher luciferase gene expression than the intronic promoter (Fig. 2B). Moreover, we found that only the activity of the host

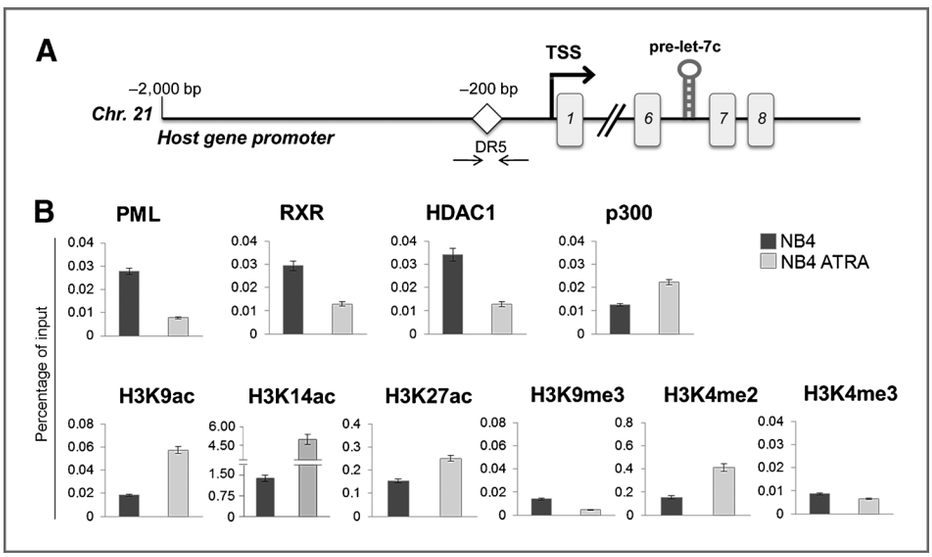

Figure 3. Epigenetic modifications of *LINC00478*/let-7c host gene promoter upon ATRA treatment. A, schematic representation of 2,000 bp upstream regulatory regions of the *LINC00478*/let-7c host gene promoter. A canonical RARE element (DR5) is indicated. Arrows outline the promoter region amplified by the specific primer pair used for qPCR amplification of immunoprecipitated chromatin. B, ChIP analysis of the *LINC00478*/let-7c host gene promoter using the indicated antibodies in NB4 cells before and after ATRA treatment (1 μmol/L, 72 hours). Results of qPCR are analyzed with the $\Delta\Delta C_t$ method. Values of each immunoprecipitated sample are expressed as percentage relative to their respective input and by subtracting the values obtained in the negative controls (no antibody). Error bars, SEM ($n = 3$).

gene promoter increased upon ATRA treatment, strongly suggesting that the higher expression levels of pri-let-7c observed in NB4 cells after ATRA (Fig. 2C) is mainly dependent upon the host gene promoter.

Epigenetic modifications of let-7c promoters upon ATRA treatment

To further investigate the involvement of the two promoters in let-7c transcription in APL, we evaluated their epigenetic status in NB4 cells before and after ATRA treatment. For this purpose, we confirmed and expanded our previous data (11), analyzing the recruitment on the host gene promoter of several additional markers of chromatin conformation (Fig. 3A and B). In particular, we found colocalization of Retinoid X Receptor (RXR) with Promyelocytic leukemia (PML) that, as expected for PML, was abolished by ATRA (36, 37 and reference therein). Moreover, ATRA induced HDAC1 removal from the promoter, accompanied by an increase of p300 histone acetyl transferase enzyme. In agreement with these data, we found a striking increase of acetylated histone H3 in K9 (H3K9ac), K14 (H3K14ac), and K27 (H3K27ac) residues, and a decrease of trimethylated H3K9 (H3K9me3), indicating a more permissive transcriptional status of the chromatin upon ATRA. On the contrary, we detected only small amount of dimethylated H3K4 (H3K4me2) and trymethylated H3K4 (H3K4me3), confirming a close chromatin configuration of this promoter in untreated cells. Surprisingly, Although ATRA administration caused an expected increase of H3K4me2, a decrease of H3K4me3 was observed (Fig. 3B). Next, we investigated the chromatin status of the proximal intronic let-7c promoter. First, we asked whether this promoter too could be a PML/RAR target. Bioinformatic analysis revealed the presence of two not canonical RARE (DR3 and ER4) located approximately 1,300 and 450 bp upstream of the TSS identified by 5′RACE (Fig. 4A). However, ChIP analysis performed around the DR3 and ER4 motifs in NB4 cells indicated that PML/RARα was recruited to a lesser extent on the intronic let-7c promoter and that ATRA did not interfere with its recruitment (Fig. 4B). We analyzed the epigenetic status of this region and found that epigenetic marks on it were generally not perturbed by ATRA treatment (Fig. 4B). To test whether other regulatory sequences, upstream of our TSS and indicated *in silico* as putative intronic let-7c promoter by others (Fig. 5A; refs. 28, 29), could epigenetically respond to ATRA, we assayed PML/RARα recruitment on and histone modifications of these regions in untreated and ATRA-treated NB4 cells (Fig. 5B–D). We found an epigenetic scenario very similar to that of our intronic promoter: PML/RARα and HDAC1 did not bind this region, and ATRA treatment did not change the bulk H3K14ac and H3K4me3, indicating that also this region is not responsive to ATRA.

Transcriptional activity of the intronic let-7c promoter in solid tumors

To assess the involvement of the host gene and intronic promoters in let-7c expression in solid tumors, we transfected the two promoter luciferase reporters in prostate (LNCaP) and lung adenocarcinoma cells (H1299). Luciferase assays showed that both host and intronic promoters are functional in these cells (Fig. 6A). However, in contrast with leukemic cells, the intronic promoter activity was higher than that of the host gene promoter. Moreover, to

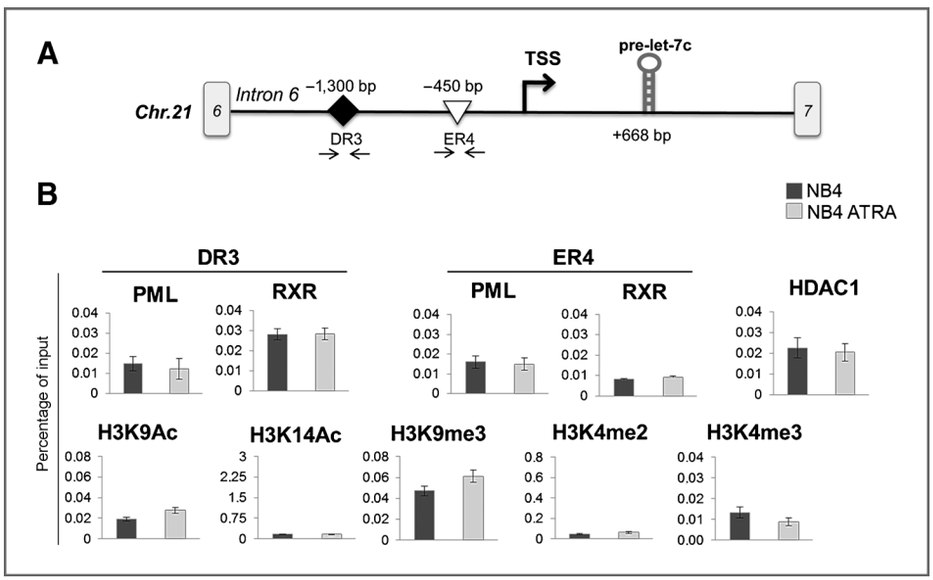

Figure 4. Epigenetic modifications of the proximal intronic let-7c promoter upon ATRA treatment. A, schematic representation of a region containing regulatory elements upstream of the pre–let-7c, identified by 5′RACE. Noncanonical RARE elements (DR3 and ER4) upstream of the let-7c initiation site are indicated. Arrows outline the position of the regions amplified by qPCR in chromatin immunoprecipitations. B, ChIP analysis of the intronic let-7c promoter using the indicated antibodies in NB4 cells before and after ATRA treatment. qPCR was performed using primers designed to amplify DNA sequences surrounding the two noncanonical RARE-binding sites DR3 and ER4 (for PML and RXR), or ER4 for the remaining antibodies. Results of qPCR are analyzed with the $\Delta\Delta C_t$ method. Values of each immunoprecipitated sample are expressed as percentage relative to their respective input and by subtracting the values obtained in the negative controls (no antibody). Error bars, SEM ($n = 3$).

better delineate the boundaries of the novel intronic promoter, we generated deletion mutants of the cloned promoter region (1,700 bp) and carried out luciferase assays. We found that the basal activity of the full-length promoter is decreased by approximately 35% by truncation mutant from −1,700 to −700 bp from the TSS (Δ#1-pGL3-prom-700), suggesting the presence of positive regulatory elements in this region. The basal activity returns at levels comparable with the full-length promoter by truncation of a further fragment (Δ#2-pGL3-prom-200; Fig. 6B), suggesting the

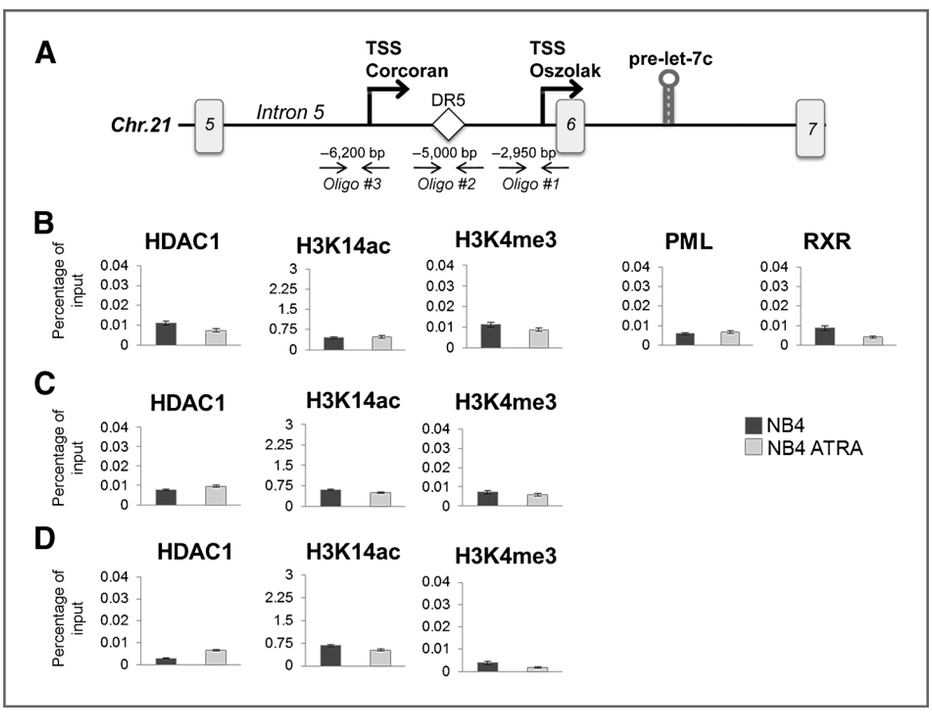

Figure 5. Epigenetic modifications of different putative intronic let-7c promoters upon ATRA treatment. A, schematic representation of putative intronic let-7c promoters predicted by Ozsolak et al. (28) and by Corcoran et al. (29). Arrows outline the position of the regions amplified by qPCR in chromatin immunoprecipitations, with the distances in base pairs (bp) respect to pre–let-7c. B to D, ChIP analysis with the indicated antibodies in NB4 promyelocytic cells before and after ATRA treatment using primers specific to the putative intronic let-7c promoter described by Ozsolak et al. (28); B, *oligo #2*; or C, *oligo #1* and by Corcoran et al. (29); D, *oligo #3*. Results of qPCR are analyzed with the $\Delta\Delta C_t$ method. Values of each immunoprecipitated sample are expressed as percentage relative to their respective input and by subtracting the values obtained in the negative controls (no antibody). Error bars, SEM ($n = 3$).

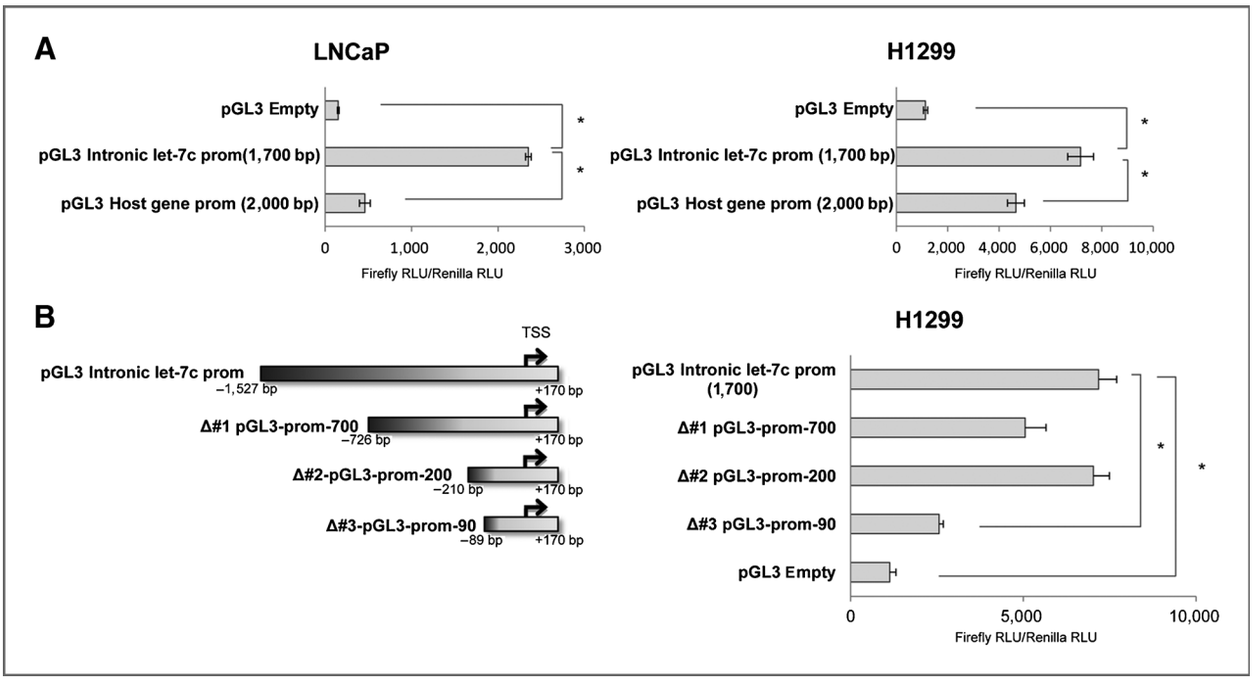

Figure 6. Transcriptional activity of the intronic and host gene let-7c promoters in solid tumors. A, Dual luciferase assay in human prostate (LNCaP) and lung (H1299) adenocarcinoma cells transfected with luciferase reporter vectors containing the intronic or host gene let-7c regulatory regions. The normalized luciferase activity, as mean of at least three independent experiments done in duplicate, is shown. Error bars, mean ± SEM ($n = 3$). *, $P < 0.05$ (Student t test). B, left, schematic representation of reporter constructs containing the −1,700 bp intronic promoter or its deletion mutants (Δ#1-pGL3-prom-700; Δ#2-pGL3-prom-200; Δ#3-prom-90). Right, dual luciferase assay in H1299 cells transfected with luciferase reporter vectors containing the intronic let-7c regulatory regions or the indicate deletion mutants. Promoter activity of each construct was determined 24 hours after transfection and luciferase activity values were normalized for transfection efficiency according to the activity of a cotransfected renilla luciferase plasmid. Data, means of ± SEM from at least three independent experiments done in duplicate. *, $P < 0.05$ (Student t test).

presence of negative regulatory elements between positions −700 and −200 from the TSS. In addition, the deletion of a fragment from −200 to −90 bp (Δ#3-pGL3-prom-90) causes a strong decrease of luciferase activity (∼75%), indicating that the minimal promoter region is approximately localized between −90 and −200 bp respect to the TSS (Fig. 6B).

Correlation of host gene and let-7c expression in different tumor histotypes.

To further investigate the relative contribution of the two promoters in different tumor histotypes, we first evaluated the correlation between the host gene and let-7c expression in AML cell lines belonging to different subtypes and in several cell lines derived from solid tumors. Because the expression of miRNAs can be regulated post-transcriptionally, in addition to mature let-7c, we measured pri-let-7c and compared the expression levels of the miRNA with those of its host gene (Fig. 7A and B). We observed that in the leukemic cell lines both pri-let-7c or mature let-7c and host gene showed coordinated expression with a Pearson correlation index of $R = 0.93$ and $R = 0.87$, respectively (Fig. 7A). On the contrary, no significant correlation was observed in several cell lines derived from different tumor histotypes (pri-let-7c $R = 0.46$; mature let-7c $R = 0.20$; Fig. 7B). Because let-7c was considered as a part of a cluster, including miR-99a and miR-125b-2, we evaluated the expression of mature and pri-miR forms of the two additional miRNAs in the locus. We show that pri-miR-99a and pri-miR-125b expression were poorly coordinated with that of let-7c ($R = 0.623$; $R = 0.618$, respectively; Supplementary Fig. S2). Moreover, we also evaluated expression of the mature form of both miRNAs and did not find correlation with mature let-7c (data not shown). These data suggest that regulation of miR-99a and -125b is at least partially independent from that of let-7c.

Levels of the histone acetylation at genomic regions around the host gene and intronic let-7c TSS in several cell lines

To further investigate this aspect, we analyzed histone H3-PAN acetylation levels in genomic regions around the host gene and intronic TSS in several cell lines with variable expression of pri-let-7c (Fig. 7B): LNCaP and SKBR-3 cells derived from metastasis of prostate and breast cancer, respectively, and representative of advanced disease; C27IM and C17IM cells derived, respectively, from clinically localized prostate cancer and normal/hyperplastic prostate (32). As shown in Fig. 7C left, we observed a peak of H3 acetylation around both the host gene and the intronic TSS in LNCaP, C27IM, C17IM, and SKBR-3, suggesting that both promoters are active. On the contrary, H3 acetylation

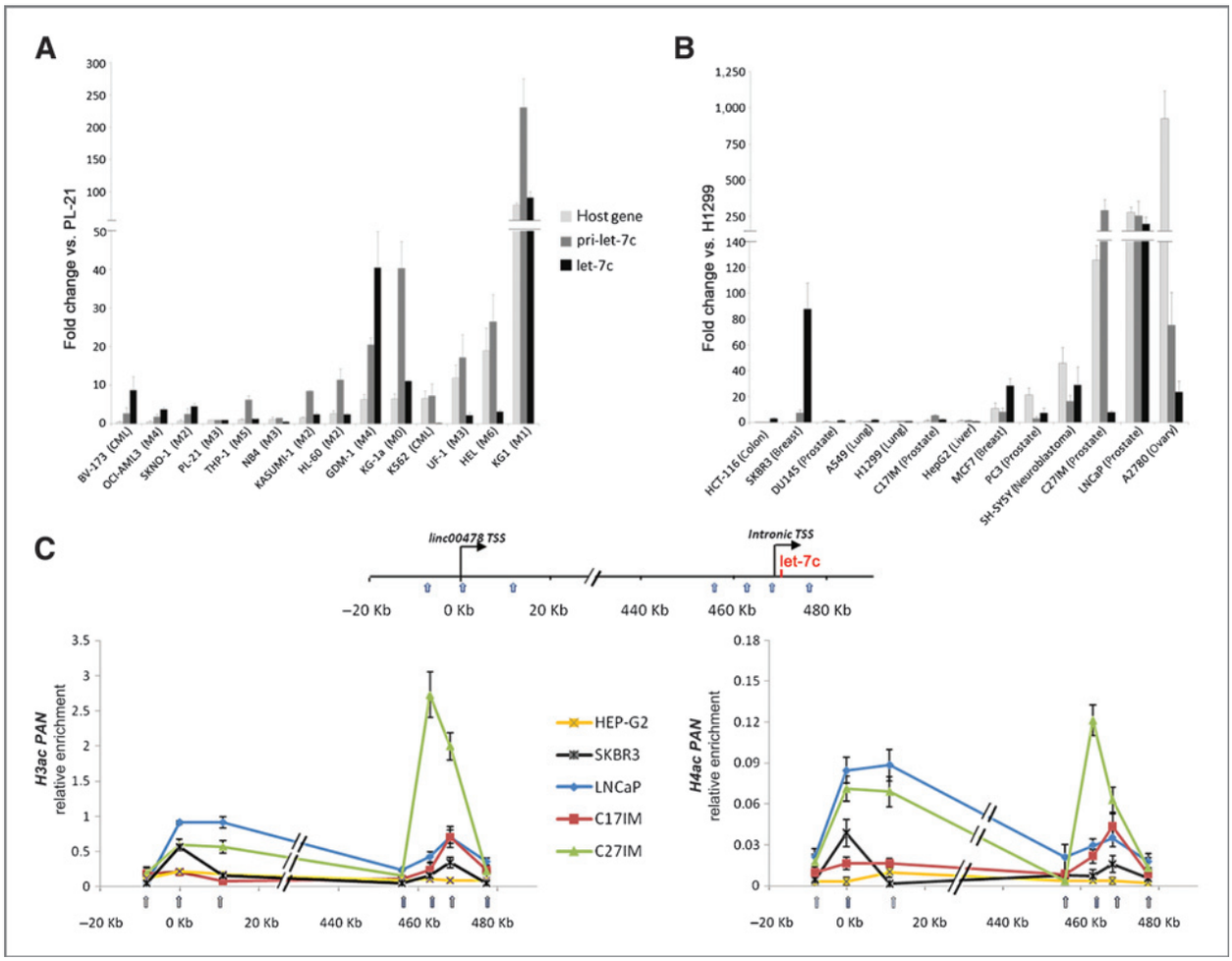

Figure 7. Expression of host gene, primary transcript and mature form of let-7c, and ChIP analysis of their promoters in cell lines from different tumor histotypes. RNA level of host gene, primary transcript, and mature form of let-7c was assessed by qRT-PCR in AML human cell lines (A) and in solid tumor cell lines (B). Results (mean ± SEM of three independent experiments) are expressed as fold changes versus PL-21 (A) or H1299 (B) cell lines. C, analysis of the host gene and intronic let-7c promoter by ChIP. Top, cartoon of the promoter region of both the host gene (*LINC00478*) and let-7c. TSS is indicated. Sky arrows, primers used for ChIP by qPCR. Arabic numbers, the length of the fragment in Kb. Bottom, ChIPs in LNCaP, C17IM, C27IM, SKBR-3, HepG2 cells using antibodies to H3 acetylation (left, H3AcPAN) or H4 acetylation (right, H4AcPAN) or no antibody. Recruitment onto host gene or intronic let-7c promoters was detected by qPCR using primers indicated by arrows in top and Supplementary Fig. S1. Results are expressed as above described. Negative controls (no antibody) values were subtracted from the corresponding sample. Error bars, SEM ($n = 3$).

was almost absent in hepatocellular carcinoma (HCC) HepG2, which was used as negative control being *LINC00478* and let-7c expression very low. Interestingly, similar results were obtained with anti-acetyl histone H4 (Fig. 7C, right).

Discussion

Let-7c is an intronic miRNA embedded in the long noncoding gene *LINC00478*. Although intergenic miRNAs genes have their own transcription regulatory elements, including promoters and terminator signals, until recently it was generally accepted that intronic miRNAs are expressed from the host gene promoter (Pol II) and require RNA splicing machinery (22–25). Previously, we observed that let-7c expression in the APL cell line NB4 was regulated in coordination with the host gene, suggesting that let-7c transcription, in this cell context, is likely controlled by the host gene promoter (11). However, processing from the host gene primary transcript may not be the only pathway for intronic miRNA generation (26–28). Several reports, using alternative strategies to predict miRNAs promoters, found many intronic miRNAs that are putatively expressed from upstream regulatory elements independently from the host gene (38 and reference therein). Bioinformatics and transcriptomic approaches has identified let-7c as a miRNA carrying a predicted PML/RARα–binding site and a region immediately upstream of the pre-miRNA sequence was considered as its putative promoter (39). In addition, others putative let-7c proximal promoters were identified combining nucleosome positioning patterns with ChIP-on-chip screens for promoter signatures (28) or by Pol II ChIP–chip data (29). In addition, Monteys and colleagues (30)

performed a genomic analysis of currently known intronic miRNA regions in the miR base database (40) and observed that approximately 35% of intronic miRNAs, including let-7c, have upstream regulatory elements consistent with promoter function. Finally, Chien and colleagues (31) identified for let-7c an intronic TSS based on high-throughput sequencing data. Although these studies strongly indicate the existence of a (or more) diverse putative intronic let-7c promoter(s), they are in disagreement for the TSS position, thus, suggesting that let-7c promoter has yet to be well defined.

In this study, we identified a novel TSS, consistent with the existence of an intronic promoter region. This new promoter has transcriptional activity, strongly supporting the hypothesis that at least two promoters have to be considered in let-7c transcription: the distal host gene and a proximal intronic promoter. Furthermore, we investigated the involvement of the two promoters in let-7c transcription in APL. Previously, we showed that PML/RARα binds canonical RARE elements in the host gene let-7c promoter in an ATRA-sensitive manner, and that its release was accompanied by an increase in H3 acetylation (11). Here, we confirmed and expanded these data, analyzing the recruitment on the host gene promoter of several additional markers of chromatin conformation. In particular, we found colocalization of RXR with PML on the host gene promoter that, as expected for PML, was abolished by ATRA (36, 37). This result is in agreement with recent findings, indicating that RXR is present within a functional PML/RARα complex (41–43). Moreover, we found that ATRA treatment leads to let-7c upregulation inducing a more open chromatin conformation of the host gene promoter, with an enrichment of epigenetic marks that correlate with a more active transcriptional state. Surprisingly, although ATRA administration caused an increase of H3K4me2, a decrease of H3K4me3 was observed. This last result seems to conflict with the other epigenetic changes, because H3K4me3 is a well-established marker of active promoters (44). However, it should be considered that the host gene promoter of let-7c lacks CpG islands, and that a different histone modifications profile, with weaker H3K4me3 signals, has been indeed associated with CpG-unrelated rather than CpG-related promoters (45). In any case, in our experimental system, this histone post-translational modification does not seem to play a major role on chromatin remodeling of the *LINC00478* host gene promoter. Next, we investigated the chromatin status of the proximal intronic let-7c promoter. Although DR5, DR2, and DR1 motifs are the most common RXR/RARα–binding sites in physiologic conditions, *in vitro* evidence suggests that the PML/RARα–containing complexes have gained an expanded DNA-binding capacity and they could bind widely spaced direct and inverted repeats as well (43–46). Bioinformatic analysis of this intronic promoter revealed the presence of two not canonical RARE sites (DR3 and ER4). However, ChIP analysis performed using specific primers surrounding the DR3 and ER4 motifs in NB4 cells indicated that PML/RARα was only weakly recruited onto the intronic let-7c promoter and that ATRA had no effect on the chromatin remodeling of this region. This latter result was confirmed for other regulatory sequences, upstream of our TSS, that emerged from diverse *in silico* analysis (28, 29) as putative intronic let-7c promoter. Moreover, in contrast with data on the host gene promoter, we found that epigenetic marks on the novel intronic promoter were generally not perturbed by ATRA treatment. Of note, a very similar scenario to that of this novel intronic promoter was found for other regulatory sequences described as putative intronic let-7c promoters (28, 29). Altogether, these results strongly support the hypothesis that in APL cells let-7c expression is mainly dependent on the host gene promoter upon ATRA-induced differentiation of APL blasts. Significantly, in contrast with leukemic cells, in solid tumors cell lines H1299 and LNCaP, the intronic promoter activity was higher than that of the host gene. These results are in agreement with several studies, indicating a specific miRNA coexpression with its host gene depending on the species, cells, disease, or even different tissues of the same disease (47). Intronic miR-483-3p, in fact, is embedded in the second intron of the insulin-like growth factor 2 (IGF-2) and is coexpressed with its host gene in most HCC tissues (38, 48); however, coexpression was not observed in a few HCC tissues because transcription from its own promoter upstream of the miR-483 gene was reported (49).

To further investigate the relative contribution of the two promoters in different tumor histotypes, we first evaluated the correlation between the host gene and let-7c expression in AML cell lines belonging to different subtypes and in several cell lines derived from solid tumors. We found that in the leukemic cell lines either the pri- or mature let-7c and host gene showed coordinated expression, whereas no significant correlation was observed in several cell lines derived from different tumor histotypes. Furthermore, we observed that the coordinated expression is higher if we consider pri-let-7c than the mature form of miRNA, i.e., for K-562, UF-1, and HEL cells (Fig. 7A). These differences could be explained by the intense post-transcriptional regulation exerted on let-7 family in certain cell types, as well described (50).

These data suggest that let-7c expression may be regulated by the host gene promoter in a wide and heterogeneous panel of tumor cells. Indeed, we observed that a high host gene expression was almost invariably accompanied with a high pri-let-7c level (Fig. 7A and B). On the other hand, the intronic promoter could play an important role in specific cell types, thus, adding a new layer in the complexity of let-7c transcription. In agreement with this analysis, we observed a peak of H3 acetylation around both the host gene and the intronic TSS in LNCaP, C27IM, C17IM, and SKBR-3, suggesting that both promoters are active. On the contrary, H3 acetylation was almost absent in HCC cells (Hep-G2) in which both *LINC00478* and let-7c expression is very low. These results reveal, for the first time to our knowledge, a potential role of the intronic promoter in let-7c transcription.

The histone marks available as a UCSC track support more the Ozsolak putative promoter than our intronic promoter. Indeed, apparently, an enrichment of H3Kme3

occurs only in the Ozsolak putative promoter. This apparent discrepancy could be due to the fact that the available data are from cell lines that are different from those we have used. Among the cells in which we observed an important role for the new identified promoter, only the LNCAP histone mark profile is reported in UCSC track. Interestingly, in this cell line an enrichment of H3Kme3 has been observed not only on the Ozsolak putative promoter but also, although to a minor extent, on our intronic promoter (Supplementary Fig. S3). Therefore, our results are qualitative comparable with that obtained in genome wide experiments.

In summary, in the present study, we found that let-7c transcription is under control of both the host gene and a proximal intronic promoter not previously identified.

Our results highlight the complexity of let-7c transcriptional regulation. Indeed, we have demonstrated that the host gene promoter is more active in a leukemic context, whereas the intronic one could play a role in others histotypes such as prostate cells.

These data strongly suggest that the expression of a miRNA involved in cell transformation is under control of different promoters and led us to propose a model in which dual or alternative promoter usage could represent a regulatory mechanism of let-7c expression in different tissues. Our results should foster detailed studies on tissue pathogenesis, which could identify let-7c promoters as novel therapeutic targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H.G. Stunnenberg, M.G. Rizzo
Development of methodology: A. Pelosi, S. Careccia, G. Sagrestani, I. Manni
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Nanni, V. Schinzari, J.H.A. Martens
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Pelosi, S. Careccia, J.H.A. Martens, A. Farsetti, H.G. Stunnenberg
Writing, review, and/or revision of the manuscript: A. Farsetti, R. De Maria, G. Piaggio, M.G. Rizzo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.P. Gentileschi, D. Del Bufalo

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