Cloning and characterization of polyA\(^-\) RNA transcripts encoded by activated B1-like retrotransposons in mouse erythroleukemia MEL cells exposed to methylation inhibitors

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We have previously identified a DNA silent region located downstream of the 3\(^{\prime}\)-end of the \(\beta\)\(^{\text{maj}}\) globin gene (designated B1-559) that contains a B1 retrotransposon, consensus binding sites for erythroid specific transcription factors and shares the capacity to act as promoter in hematopoietic cells interacting with \(\beta\)-globin gene LCR sequences \textit{in vitro}. In this study, we have cloned four new non-polyA RNA transcripts being detected upon blockade of murine erythroleukemia (MEL) cell differentiation to erythroid maturation by methylation inhibitors and demonstrated that two of them share high structural homology with sequences of B1 element found within the B1-559 region. Although it is not clear yet whether and how these RNAs interfere with induction of erythroid maturation, these data provide evidence for the first time showing that methylation inhibitors can activate silent repetitive DNA sequences in MEL cells and may have implications in cancer chemotherapy using demethylating drugs as antineoplastic agents. [BMB reports 2012; 45(2): 126-131]

INTRODUCTION

Previous work from our laboratory has indicated that induction of hemoglobin synthesis and terminal erythroid maturation in MEL cell is associated with changes in methylation of both polyA\(^+\) and polyA\(^-\) RNA as well as with alterations in the intracellular concentration of SAH/SAM ratio involved in the active methylation cycle (1, 2). Such a conclusion was further supported by the observation that N6-methyladenosine (N6mAdo) inhibits commitment of MEL cells to terminal maturation induced by chemical inducers through its intracellular conversion into S-N6-methyladenosylhomocysteine (N6-SAHA), an active intermediate that affects methylation of RNA. The potential role of changes in RNA methylation was further strengthened by data showing that pharmacological agents which inhibit active methylation cycle block MEL cell differentiation program \textit{in vitro} and trigger the cytoplasmic accumulation of relatively short RNA transcripts lacking polyA-tail (1-4). Molecular hybridization mapping analysis has revealed that these RNAs are likely to be encoded by DNA sequences located downstream from 3\(^{\prime}\)-end of \(\beta\)\(^{\text{maj}}\) globin gene. To understand the role of these RNA transcripts in the blockade of MEL erythroid maturation, it was critical to clone and characterize these RNA species and identify their sequences and origin.

In this study, we have structurally characterized the 3\(^{\prime}\)-flanking \(\beta\)\(^{\text{maj}}\) globin DNA sequences that presumably encode polyA RNA transcripts accumulated upon blockade of MEL cell differentiation with methylation inhibitors by cloning and characterizing these molecules. To this end, we have carried out two sets of studies. In the first, we made an effort to map DNA sequences located downstream from the 3\(^{\prime}\)-end of \(\beta\)\(^{\text{maj}}\) globin gene that presumably encode the polyA RNA transcripts and in the second, to clone and further characterize the relatively low molecular weight RNA transcripts. These studies revealed a DNA region of 559 bp (designated B1-559) that contains several ATG initiation codons, a B1-retrotransposon element and shares structural homology with at least two RNA species (151 and 448 nt) out of four RNA transcripts cloned (151, 174, 391 and 448 nt) upon treatment of MEL cells with methylation inhibitors. Recently, we have demonstrated the capacity of B1-559 to recruit binding of nuclear trans-acting proteins derived from MEL cells as well as to activate transient expression of a reporter gene in hematopoietic cells either alone or in cooperation with DNase I hypersensitive site 2 (HS2) sequences of \(\beta\)-globin gene LCR enhancer (5). The implication of methylation inhibitors-induced activation of B1-like silent DNA regions in patients undergoing cancer chemotherapy using hypomethylating agents as antineoplastic agents are discussed.

Keywords: B1 retrotransposon element, Erythropoiesis, MEL cell differentiation, Methylation inhibitors, Non-polyA RNAs, Repetitive DNA, Silent DNA sequences
RESULTS

Mapping of the 3′-end downstream flanking βmajor globin gene region to locate the origin of short RNAs detected in MEL cells co-exposed to DMSO and/or a methylation inhibitor

To analyze both structurally and functionally the 3′-end downstream flanking βmajor globin sequences and demonstrate or rule out the possibility whether the polyA RNA transcripts originate from this DNA region, an experimental strategic approach was applied that aimed to: a) map the region of possible origin of RNA species; b) uncover the protected RNA species by S1 nuclease mapping and confirm their origin of transcription; and c) clone and identify RNA transcripts via RT-PCR and subsequent sequencing of cloned cDNAs.

At first, four DNA probes were generated (Fr1, Fr2, Fr3 and Fr4) by PCR covering the 3′-flanking βmajor globin gene region being under analysis (Fig. 1A) for subsequent use in Northern blot hybridization analysis experiments to locate the origin of RNA molecules. Interestingly enough, the molecular hybridization revealed that only DNA probes Fr1 (Fig. 1B), Fr2 (Fig. 1C) and Fr3 (Fig. 1D) but not Fr4 (Fig. 1E) allowed the detection of short-ended RNA transcripts. Such a finding suggested that the origin of RNA species might be located adjacent to the B1 element previously found at the 3′-flanking βmajor globin gene DNA sequences (see Fig. 1A) (4). Subsequently, nuclease-S1 mapping was carried out to assess RNA molecules being protected by the 7.304 bp genomic DNA fragment when cytoplasmic RNA derived from MEL cells exposed to methylation inhibitors was hybridized. Following this direction, the presence of βmajor globin mRNA and another lower molecular weight RNA was confirmed (Fig. 2B). The βmajor globin mRNA was protected from nuclease-S1 degradation, although it was detected at lower molecular weight as compared to the original intact molecule. This result should be interpreted by the existence of polyA-tail added post-transcriptionally in the intact βmajor globin mRNA.

Cloning and characterization of RNA transcripts accumulated in the cytoplasm of MEL cells exposed to methylation inhibitors

As we have shown previously, exposure of MEL cells to neplanocin A, 3-deazaanplanocin A and cycloleucine specifically blocks erythroid differentiation program and leads to cytoplasmic accumulation of relatively small size polyA RNA transcripts (1-4). Consequently, the next experimental task was then the identification of such short-ended RNA transcripts via cloning and sequencing. Despite the technical difficulties experienced at the beginning of this study, we were able to clone four RNA molecules by RT-PCR rapid cloning using primers derived from DNA sequences located downstream from the 3′-flanking βmajor globin gene region (B1-559) (Fig. 1A). Indeed, four RNA species were amplified in RT-PCR when cytoplasmic RNA isolated from DMSO-treated MEL cultures in the presence of neplanocin A for 72 hr was used (Fig. 3A). To confirm the integrity of cytoplasmic RNA and the quality of the synthesized cDNAs, additional PCR was performed by using specific pair of primers able to amplify the developmentally regulated βmajor globin (Fig. 3B) and the housekeeping β-actin (Fig. 3C) genes. The size of the identified RNA molecules corresponding to the cloned DNA fragments isolated by RT-PCR has been 448, 391, 174 and 151 nt in length (the accession numbers of these clones in Gen/EMBL databank are FM992847, FM992848, FM992849, FM992850, respectively). Searching in DNA sequence databanks revealed that these RNA molecules share structural homology with DNA sequences located in mouse genome at chromosome 3 for 448 nt (similarity 99%; NT_039240.7/Mm3_39280_37; position: 44472674-44473110), at chromosome 5 for 391 nt (similarity 98%; NT_078458.6/Mm5_78523_37; position: 9624002-9623626), at chromosome 9 for 174 nt (similarity 97%; NW_001030907.1/Mm9_11172196_37; position: 34369047-34369207) and at chromosome 3 for 151 nt.

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Fig. 2. Assessment of the products of nuclease S1 mapping reactions by Northern blot hybridization using as probe the 7.304 bp genomic DNA fragment. The nuclease S1 mapping reactions (lanes 3-7) were performed as indicated under "Materials and Methods" by using different units of nuclease S1 in each separate assay shown in (B) as follows: 100 units (lane 3), 200 units (lane 4), 300 units (lane 5), 400 units (lane 6) or 500 units of the enzyme (lane 7). Constant amount (10 μl) from each individual assay was used for further assessment by Northern blot hybridization analysis using [32P]-labeled 7.304 bp genomic DNA fragment bearing the \( \beta \) major globin gene shown in Fig. 1A. Ethidium bromide staining patterns of electrophoresed RNAs is shown in (C, D). In (A), the isolated RNA samples used to detect the \( \beta \) major globin mRNA and polyA RNA transcripts is shown.

Fig. 3. RT-PCR analysis of cytoplasmic RNA transcripts isolated from MEL cell cultures. Samples of total cytoplasmic RNA shown in Fig. 1 was isolated and subjected (1 μg) to RT-PCR analysis as shown under "Materials and Methods" (A). Note that the same RNA samples were also subjected to RT-PCR analysis using a pair of primers specific for either exon-2 of \( \beta \) major globin mRNA (B) or for mouse \( \beta \)-actin (C) mRNAs to check the quality of isolated RNA transcripts. The alignment of the 5'-end of the cloned 448 and 151 nt RNA transcripts with the corresponding 5'-end DNA sequences of the B1 element located in the 3'-end flanking \( \beta \) major globin gene locus (its position is depicted in Fig. 1) is shown in (D, E), respectively.

Expression profile analysis of the cloned 448 nt RNA transcript in control and differentiating MEL cells exposed to methylation inhibitors

Based on the previous data showing that the cloned 448 nt RNA transcript share common DNA sequences with the B1 element in high homology and the fact that the detection of RNA species in the cytoplasm of MEL cells treated with methylation inhibitors was achieved only with probes derived from the vicinity of the B1 element (Fig. 1A) it was reasonable to focus on this RNA molecule and ask if its expression profile in MEL cells exposed to methylation inhibitors is somehow correlated with the steady-state level of RNA transcripts detected by Northern blot hybridization analysis (Fig. 1B, C and D). To this direction, we used the 448 nt RNA molecule to generate [32P]-labeled probes and carried out Northern blot experiments to assess cytoplasmic RNA transcripts derived from control and differentiating MEL cells exposed to different methylation inhibitors.

Expression profiles showed that the expression of the cloned 448 nt RNA transcript in MEL cells was significantly higher than in control cells (Fig. 3A). In particular, the 448 nt cloned RNA transcript shows low expression in parental control-untreated MEL cells, not detectable levels in neplanocin-treated and high level of expression in DMSO-treated that was further enhanced upon co-treatment of cells with DMSO and neplanocin for 72 hr. On the other hand, only the cloned 151 nt RNA transcript exhibited hardly detectable levels in DMSO-treated cells, while the other two 391 nt and 174 nt RNAs retained detectable expression levels only upon co-treatment of MEL cells with DMSO and neplanocin.

Another interesting point that emerged from these RT-PCR experiments is the fact that, among the cloned RNAs, variability in their steady-state level exists among the different treatment conditions employed in MEL cell cultures. To this regard, it has to be noticed, that mainly the concomitant exposure of MEL cells with DMSO and neplanocin A activated to a greater extent the expression level of such RNAs (Fig. 3A). In particular, the 448 nt cloned RNA transcript shows low expression level in parental control-untreated MEL cells, not detectable levels in neplanocin-treated and high level of expression in DMSO-treated that was further enhanced upon co-treatment of cells with DMSO and neplanocin for 72 hr. On the other hand, only the cloned 151 nt RNA transcript exhibited hardly detectable levels in DMSO-treated cells, whereas the other two 391 nt and 174 nt RNAs retained detectable expression levels only upon co-treatment of MEL cells with DMSO and neplanocin.
represent members of a class of polyA- RNAs transcripts with the original cloned 448 nt RNA transcript and thus they could detected three RNA transcripts share structural similarity with their level enhanced after 72 hr. These data suggest that the hr exposure of MEL cells to methylation inhibitors, whereas inducer DMSO for less than 72 hr (Fig. 4B, C and E). At least three RNA transcripts were detected in the cytoplasm after 48 hr exposure of MEL cells to methylation inhibitors, whereas their level elevated after 72 hr. These data suggest that the detected three RNA transcripts share structural similarity with the original cloned 448 nt RNA transcript and thus they could represent members of a class of polyA' RNAs transcripts with core structural domain sequences. Such a possibility has been already shown to exist between B1 and the two cloned RNAs (448 nt and 151 nt; Fig. 3D and E) and also proposed by S1 nuclease experiments (Fig. 2B).

![Diagram](image)

**Fig. 4.** Assessment of the effect of neplanocin A, 3-deazaneplanocin A and cycloleucine on the accumulation of discrete RNA transcripts, in control and differentiating MEL cells. MEL-745PC-4A cells were incubated in culture in the absence or presence of DMSO (1.5 % v/v) or exposed separately to neplanocin A (1 × 10^{-5} M), 3-deazaneplanocin A (1 × 10^{-5} M) and/or cycloleucine (4 × 10^{-5} M) in the presence or absence of DMSO. At times indicated (numbers above the panels), total cytoplasmic RNA was isolated from cultures and used (10 μg) to perform Northern blot hybridization analysis using the DNA probes shown in (A) as follows: 448 bp (B), 122 bp (C), or 300 bp (E). In these experiments, the housekeeping gene GAPDH (F) was used as control (4). In (D, G) the ethidium bromide staining pattern of electrophoresed RNAs is shown. Note that the DNA probes generated from the cloned 448 nt RNA molecule are diagrammatically shown in (A); Sdr: short direct repeats.

**DISCUSSION**

Although human cancers are characterized by widespread changes in their genomic DNA methylation and histone modification patterns, the clinical proof-of-concept and specificity for epigenetic cancer therapies remains to be clearly established (6). Furthermore, it has long been known that in cancer cells repetitive DNA elements suffer abnormal demethylation with potential loss of their silencing status. Interestingly, it has been shown that the activity of L1s during brain development and their contribution to neuronal function by modulating gene expression is mediated through the pivotal role of methyl-CpG-binding protein 2 (MeCP2) by acting as a modulator of L1s neuronal transcription and retrotransposition (7). To this regard, the pharmacological activity for demethylating drugs is accompanied by a pleiotropic response involving modulation of expression of a large number of genes also implying a lack in target specificity (8). This evidence is further supported by the fact that besides the known cancer cell heterogeneity, unique pattern changes in the DNA methylation levels of individual repetitive DNA elements have been recorded in healthy persons as well as among patients suffering from bladder cancer, CML and/or acute promyelocytic leukemia (APL) (9). Also, by using genome-wide microarray approaches to assess methylation level of DNA repetitive elements in patients with head and neck cancers, it was able researchers to show that significant changes exist with loss of DNA methylation to be most pronounced for certain repeat sequences family members (10). This means that, upon treatment of cancer patients with demethylating drugs to restore pathological epigenetic changes, the pharmacological effect can vary significantly for each repetitive element also impinging on their function for transcriptional gene activation and/or silencing. Recently, an important paradigm for demethylation-dependent modulation of gene expression involving repetitive elements has been revealed. Indeed, azacytidine and decitabine has altered cMet proto-oncogene expression in colon carcinoma and myeloid leukemia cells through demethylation of an antisense promoter located within a LINE-1 element being part of the second intron of the cMet gene (11). Similar results were also obtained in bladder tumors, where LINE1 promoter hypomethylation contributed to chromosomal instability and altered the functional cancer cell transcriptome. In this case, hypomethylation of LINE-1 promoter has also activated an alternate transcript of cMet, a result that may be related to bladder cancer predisposition and disease progression (12).

MEL cell differentiation system has been proven a useful model to uncover cellular and molecular events of erythropoiesis involved in leukemic cell maturation in vitro (13-15). The promoter-like activity of B1-559 DNA region recently shown (5) and the observed structural similarity of the cloned RNA transcripts with the B1 element located within this locus extend our knowledge on how non-coding RNA molecules derived from genomic repetitive sequences can be generated, un-
under conditions where MEL cells are blocked to differentiation by the action of methylation inhibitors. These data are also quite interesting in the light of evidence that methylation inhibitors, like 5-azacytidine (azacitidine), 5-aza-2'-deoxycytidine (decitabine) and pyrimidine-2-one β-ribofuranoside (zebularine), currently in use for the therapy of myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML), exhibited differential hypomethylating capacity in LINE1 DNA repeat sequences as well as in activating genes relevant to leukemiaogenesis (16). At present we have no clue what is the precise function of the cloned small RNAs and if are involved in blocking the commitment of MEL cells to erythroid maturation. Alternatively, one can ask whether these RNAs act at transcriptional or even posttranscriptional level to abrogate MEL cell erythroid differentiation. Whether this region plays any role and how upon transcriptional regulation of β-globin gene locus remains to be elucidated. Alternatively, and based on the capacity of B1-559 to act as a potential promoter in erythroid hematopoietic cells as shown in another study (5), we can postulate that B1-559 unmasked by chromatin remodeling processes can act developmentally upon β-globin gene transcriptional regulation by affecting specific LCR and promoter interactions. To this regard, the cytoplasmic accumulation of the cloned RNA transcripts upon exposure of MEL cells with methylation inhibitors imply that hypomethylation of DNA is at least one mechanism underlying their transcriptional activation. Conversely, the interplay between repetitive elements with transcription factors, DNA methylation and even miRNA function tend to suggest that these elements may contribute to specific transcription-related circuits to modulate gene expression upon development and evolution. If such a molecular circuit exists in our case, where B1-559 sequences and the cloned B1-like RNA transcripts (448 nt and 151 nt) confer somehow their function to other gene regulatory factors, it remains to be documented. In any case, however, the ability of methylation inhibitors to activate previously silent genomic regions, such as B1-like elements, represents an interesting observation of pharmacological and clinical value on light of current cancer chemotherapy with DNA demethylating drugs being in use or under development.

MATERIALS AND METHODS

Cell cultures, assessment of differentiation, isolation of cytoplasmic RNA, Northern blot hybridization analysis and S1-nuclease RNA mapping assay

The growth of MEL-745PC-4A cells, assessment of erythroid differentiation in vitro, isolation of cytoplasmic RNA from cultures and subsequent Northern blot hybridization analysis were carried out as described elsewhere (2, 4). Furthermore, the S1 nuclease protection assay was carried out as previously described (17).

RT-PCR for the generation of specific DNA fragments derived from 3'-flanking βmajor globin sequences used in Northern blot hybridization analysis

The pBluescript (+7.3 kb) plasmid bearing the 7,304 bp genomic DNA fragment that contains the βmajor globin gene (accession number X14061 at Gen/EMBL databank) was used as template for the generation of DNA probes consisting of fragments Fr1 (1,841 bp), Fr2 (543 bp), Fr3 (203 bp) and Fr4 (1,256 bp) shown in Fig. 1A by using the following pair of primers: forward primer for both DNA probes Fr1 and Fr2: 5'-CGGCTCGAGATGCCCAGACTTGGCAAATTAG-3', with reverse primer for Fr1, 5'-CTCTCATTACATCTGTC-3' and reverse primer for Fr2, 5'-ggaagactTACGACATCTTCCAAGTAC-3'; For DNA probe Fr3: forward primer: 5'-AAAAGGAGTCTCATGT CCAGGGCG-3' and reverse primer: 5'-CTGTCAAGATATGAGAC TCCT-3'; For DNA probe Fr4: forward primer 5'-CAGGAGTTC CATATTGAAAG-3' and reverse primer 5'-CTCTACATTTACA TCACTGTC-3'. The low-case letters in primer sequences represent specific restriction enzyme sites added to facilitate subsequent cloning. All the PCR products were purified and verified by DNA sequencing.

RT-PCR cloning of RNA transcripts isolated from MEL cells treated with methylation inhibitors

RT reaction (at 37°C for 90 min) was carried out to generate cDNAs from 10 μg of total cytoplasmic RNA isolated from either untreated parental MEL or MEL cells incubated with DMSO (210 mM) in the absence or presence of neplanocin A (1 x 10⁴ M) for 72 hr by using 10 μl (0.5 μg/μl) random hexamer primers, and reverse transcriptase (220 U). These cDNAs were purified (PCR purification kit; Qiagen) and then used as templates in PCR to detect any DNA fragments generated by using the following primers derived from the B1-559 downstream b major globin gene DNA region:. Forward primer: 5'-AAAAGGAGTCTCATGTCCAGGGCG-3'; reverse primer: 5'-CTGC TGACTATGAGACTCCT-3'. The PCR products were cloned into pCR2.1 vector, sequenced and the data submitted to Gen/EMBL databank under accession numbers FM992847, FM992848, FM992849 and FM992850. For subsequent Northern blot hybridization experiments, the cloned 448 bp DNA fragment was used as template in PCR to generate two smaller DNA fragments of 122 bp and 300 bp length by using suitable pair of primers as follows: Forward primer: 5'-GAGGAGCTGTATTGAAAT GCCGCAGCC-3'; reverse primer 5'-AAAAGGAGTCTCATGAGCG-300 bp DNA fragment); forward primer: 5'-AAAAGG AGTCTCATGACGCAGC-3'; reverse primer 5'-CTGC TGACTGAGAC-122 bp DNA fragment). The generated PCR products were then purified and verified by DNA sequencing.

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