Transcription-Coupled Translation Control of AML1/RUNX1 Is Mediated by Cap- and Internal Ribosome Entry Site-Dependent Mechanisms

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AML1/RUNX1 belongs to the runt domain transcription factors that are important regulators of hematopoiesis and osteogenesis. Expression of AML1 is regulated at the level of transcription by two promoters, distal (D) and proximal (P), that give rise to mRNAs bearing two distinct 5′ untranslated regions (5′UTRs) (D-UTR and P-UTR). Here we show that these 5′UTRs act as translation regulators in vivo. AML1 mRNAs bearing the uncommonly long (1,631-bp) P-UTR are poorly translated, whereas those with the shorter (452-bp) D-UTR are readily translated. The low translational efficiency of the P-UTR is attributed to its length and the cis-acting elements along it. Transfections and in vitro assays with bicistronic constructs demonstrate that the D-UTR mediates cap-dependent translation whereas the P-UTR mediates cap-independent translation and contains a functional internal ribosome entry site (IRES). The IRES-containing bicistronic constructs are more active in hematopoietic cell lines that normally express the P-UTR-containing mRNAs. Furthermore, we show that the IRES-dependent translation increases during megakaryocytic differentiation but not during erythroid differentiation, of K562 cells. These results strongly suggest that the function of the P-UTR IRES-dependent translation in vivo is to tightly regulate the translation of AML1 mRNAs. The data show that AML1 expression is regulated through usage of alternative promoters coupled with IRES-mediated translation control. This IRES-mediated translation regulation adds an important new dimension to the fine-tuned control of AML1 expression.

The AML/RUNX genes (previously termed CBFA or PEBP2α [61]) belong to a gene family of heterodimeric transcription factors. Family members encode a protein with a highly conserved region of 128 amino acids designated the runt domain (RD), because of its homology to a region in the Drosophila Runt protein (21). The RD mediates both AML heterodimerization with the CBFB protein and binding of AML/RUNX to its consensus DNA sequence, PyGPyGGT (17, 21, 36, 61). In humans and mice, three highly conserved AML genes have been identified: AML1/RUNX1 on chromosome 21q22.1, AML2/RUNX3 on chromosome 1p36, and AML3/RUNX2 on chromosome 6p21 (16, 31). Homozygous disruption of AML1/RUNX1 and AML3/RUNX2 in mice indicated that AML1/RUNX1 plays a crucial role in hematopoiesis (44, 45, 69) while AML3/RUNX2 is essential for osteogenesis (51). Importantly, the AML1/RUNX1 and CBFB genes are the most frequent targets for leukemia-associated translocations (32), further highlighting the pivotal role these genes play in hematopoiesis. AML1/RUNX1 is abbreviated AML1 in this paper.

Transcription of AML1 is initiated at two promoter regions, designated the proximal (P) promoter and the distal (D) promoter (12). Genomic analysis revealed that the distance between them is ~150 kb (Fig. 1) (unpublished data). Transcription yields mRNAs that differ in their 5′ untranslated region (5′UTR), the transcripts carry either the D-UTR or the P-UTR (Fig. 1) (12). The P-UTR is remarkably long (1,631 bp) but is nevertheless carried on a single exon. It contains 15 AUG codons upstream of the authentic initiator AUG (uAUG), several of which are followed by short open reading frames (ORFs) (29). Such uAUGs were shown to inhibit translation initiation (23). Two GC-rich islands, which could form stable stem-loop structures, are present at the 5′ and 3′ parts of this 5′UTR (29). These features suggested that translation of P-UTR-bearing mRNAs via the ribosome-scanning mechanism would be inefficient (24). The D-UTR, while much shorter than the P-UTR, consists of four exons that are alternatively spliced (Fig. 1). When all four exons are included, the length of the D-UTR adds up to 452 bp. It contains only two uAUGs and lacks GC-rich elements. The striking differences in size and structure between the two UTRs and the presence in the P-UTR of structural elements characterizing an internal ribosome entry site (IRES) (reviewed in reference 18) prompted us to investigate the possibility that AML1 expression is regulated at the level of mRNA translation.

Since the initial identification of IRES regions that allow cap-independent translation in picornavirus mRNAs (19, 47), IRES elements have been found in several cellular mRNAs including those encoding the human immunoglobulin heavy-chain binding protein (BiP) (33), the Drosophila Antennapedia and Ultrabithorax proteins (70), fibroblast growth factor 2 (FGF2) (67), platelet-derived growth factor 2 (PDGF-2/c-sis) (4), insulin-like growth factor II (IGF-II) (65), translation initiation factor eIF4G (11), human c-myc (42, 63), cardiac voltage-gated potassium channel Kv1.4 (43), nervous system-specific DNA-binding protein-MYT2 (22), vascular endothelial
expression of AML1 is strictly regulated during development and in adult life, but surprisingly little is known about the molecular mechanisms regulating the expression of the gene. In mouse embryos it is expressed in a number of tissues (44, 57), while in adults it is expressed mainly in the hematopoietic system (40, 55). Here we demonstrate that AML1 expression is regulated through usage of alternative promoters coupled to translation control by either cap- or IRES-dependent mechanisms.

MATERIALS AND METHODS

Plasmid constructions. Plasmid M-CAT (pOS14) (5) contains the Escherichia coli chloramphenicol acetyltransferase (CAT) gene, placed between the bacteriophage T7 promoter and transcription terminator. The unique NcoI site of pOS14 is located downstream of the T7 promoter and harbors the translational initiation codon of the CAT coding sequence. Plasmids T7-MAP and T7-MAD were constructed by inserting an NcoI fragment of 1,631 or 452 bp, containing the proximal (P) or distal (D) AML1 5’UTRs, respectively, into the NcoI site of pOS14. This resulted in placement of the 5’ UTR segments between T7 promoter and the CAT gene in both orientations. The 5’ UTR fragments were generated by PCR, using plasmids containing the relevant 5’ UTR (29) as the template and the sense oligonucleotide primer 5’-GGCGCCCGATGGCCGCAAGCAGG-3’ (T7-MAP) or 5’-AAAAACCCATGGAACCAAGTTGGTGAGC-3’ (T7-MAD) and the antisense oligonucleotide primer 3’-GAAACAGCTTACCATCGAGAGATG-3’. The sense oligonucleotide primer 3’-GAAACAGCTTACCATCGAGAGATG-3’ and the antisense oligonucleotide primer 5’-GGCGCCCGATGGCCGCAAGCAGG-3’ were inserted in the pBS-AML1/Apa site at the initiation codon of the CAT coding sequence. Plasmids T7-MAP and T7-MAD were constructed by inserting a 935-bp fragment of pBS-SK, digested with NcoI and SmaI, containing a bicistronic unit composed of CAT and LUC ORFs separated by the encephalomyocarditis virus (EMCV) 5’UTR and driven by the T7 promoter. A PCR fragment was digested with EcoRI and XbaI, and inserted into the XhoI site of pEGFP-N1 (Clontech Laboratories, Palo Alto, CA), resulting in the expression of both CAT and LUC ORFs. The PCR fragment was generated by PCR using as a template 5’-GGCGCCCGATGGCCGCAAGCAGG-3’ and 3’-GGAAGGATGCGGTAGTGAGC-5’ with an internal NcoI site at the initiation codon of the CAT coding sequence. The 935-bp fragment was inserted into the pBS-AML1/Apa site at the initiation codon of the CAT coding sequence. The resulting recombinant plasmid was linearized by filling in the NcoI sites with DNA polymerase I and religation using T4 DNA ligase.

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Cells were scored as benzidine positive (blue) or benzidine negative (yellow) using a Leitz-Wetzlar microscope at a magnification of $\times 100$.

**Transfection into cell cultures.** Plasmid DNAs were prepared using the Qiagen plasmid purification kit. Exponentially growing SV80, HeLa, or 293 cells were transfected with plasmid DNA (15 µg for SV80, 25 µg for HeLa, and 2.3 µg for 293) using the standard method of calcium phosphate-mediated transfection followed by incubation for 48 h at 37°C prior to analysis.

**Electroporation.** Electroporation was employed for the hematopoietic cell lines. Cells (2 $\times$ 10$^7$) in log phase were transfected using 25 µg of plasmid DNA as specified by the manufacturer (BTX), using a capacitor discharge of 250 V, 1.700 µF, and 13 Ω (R1) for K562 cells or 250 V, 1.700 µF, and 72 Ω (R4) for Jurkat, K562, SUP-T1, B9, and SKW6.4 cells were electroporated as above (but without addition of saline sperm and HEBS buffer), with a capacitor discharge of 110 V, 2,750 µF and 129 Ω (R5) for SUP-T1 and B9 cells and 250 V 1,700 µF, and 13 Ω (R1) for SKW6.4 cells. Following electroporation, the cells were immediately transferred to 30 ml of growth medium and incubated for 48 h prior to analysis.

**Cell extracts.** At 48 h after treatment, cells were collected, washed with phosphate-buffered saline (Ca and Mg free), and centrifuged. Cell pellets were resuspended in 0.1 ml of 0.1 M Tris-HCl (pH 8.0) and lysed by four freeze-thaw cycles (30 s in liquid N$_2$, 1 to 2 min at 30°C in a water bath). Suspensions were centrifuged at room temperature, and supernatants were assayed for LUC and CAT activities. LUC signals were monitored with a TD-200 luminometer (Turner). CAT activity was determined by a phase extraction assay and counted in the $^{14}$C channel of a 1500-TIR-CARB liquid scintillation analyzer (Packard) as described previously (4).

**Protein and RNA analysis.** Cells were harvested and washed once in phosphate-buffered saline, and proteins or RNAs were extracted as described previously (3). Protein extracts were subjected to Western blot analysis as described previously (4). Poly(A)$^+$ RNA was purified from 150 µl of total RNA using oligo(dT) magnetic beads (Dynal, Oslo, Norway) and then was subjected to Northern blot analysis as previously described (29).

### RESULTS

**Expression of AML1 is modulated in vivo by P-UTR and D-UTR.** AML1 expresses several size classes of mRNAs (Fig. 2A, left). Transcription of the 6-kb species is regulated by the D promoter, and hence this species carries the shorter (0.45-kb) D-UTR, while the 8- and 4-kb mRNAs are transcribed via the P promoter and carry the long (1.6-kb) P-UTR (Fig. 1A) (12). In addition to differential promoter usage, the various AML1 mRNAs are generated through alternative splicing and by the use of spaced polyadenylation signals. The difference in transcript length results mainly from a different combination of UTRs (29). The initiator AUG codon in mRNAs bearing the D-UTR resides in exon 1d, while in mRNAs harboring the P-UTR it is in exon 3 (Fig. 1A). Hence, proteins translated from the 6-kb mRNAs containing the D-UTR possess an additional 32 amino acids, encoded by exons 1d and 2, that are not included in the 8- and 4-kb mRNAs generated from the P-promoter (Fig. 1). To distinguish between these two classes of protein products, we used antibodies raised against the 17-amino-acid peptide encoded by exon 1d (designated α-distal) (Fig. 1B). The other type of antibodies (α-TD) are directed against the transactivation domain (TD) of AML1 (3) and were used to detect all the isoforms that contain the TD.

Northern analysis of RNA from the human erythroblast cell line K562 and the human B-lymphoblast cell line Daudi is presented in Fig. 2A, left. K562 cells express predominantly the 8- and 4-kb mRNA species, transcribed by the P-promoter, while in Daudi cells the major mRNA was the D-promoter-directed 6-kb mRNA (Fig. 2A, left). Parallel analysis of the protein products showed a very small amount of AML1 proteins in K562 and a relatively large amount of the protein of the expected size (53 kDa) in Daudi cells (Fig. 2A, right). Importantly, the AML1 proteins in Daudi cells reacted with both the α-TD and α-distal antibodies, indicating that they were translated from mRNAs containing the D-UTR. These data are consistent with the possibility that the P-UTR-containing mRNAs (the 8- and 4-kb species) in K562 were poorly translated compared to the D-UTR-containing mRNA (the 6-kb species) in Daudi cells. Differential levels of AML1 D- and P-proteins were also seen in thymocytes and blood lymphocytes (Fig. 2B). Thymocytes expressed exclusively the D-UTR-bearing mRNAs (lane b), which gave rise to AML1 proteins containing the distal peptide (lanes d and f). Lymphocytes and human umbilical vein endothelial cells (HUVEC), on the other hand, expressed mainly P-UTR-containing mRNAs (the 8-, 4- and 3-kb species) (lanes a and c), which gave rise to a low level of the P-protein (lanes 3 and g). Expression of AML1 was also affected by various exogenous stimuli. Incubation of fresh human lymphocytes for 48 h under culture conditions caused a promoter switch linked to a marked increase in the 6-kb mRNA species (Fig. 2C, lanes a and b) and a consequent increase in the production of the 53-kd AML1 protein (compare lanes d and e and compare lanes g and h). Mitogenic stimulation with tetradeacanylophor acetate and concanavalin A (TPA/ConA) abolished transcription of the 6-kb mRNA (compare lanes b and c) and reduced the production of the 53-kd protein (lanes e and f and lanes h and i). Taken together, the data show that AML1 expression is regulated in vivo, in different cell types and in response to physiological stimuli, at both the transcriptional and posttranscriptional levels. This raised the possibility that the two 5′-UTRs are involved in translation regulation of AML1 expression.

Transcripts bearing the P-UTR are poorly translated in vitro, while mRNAs bearing the D-UTR are readily translatable. To investigate the involvement of the P- and D-UTRs in translation control of AML1, the translation efficiency of the two UTRs was first assayed in vitro. The P-UTR and D-UTR were cloned into the CAT expression vector between the T7 promoter and CAT to generate monocistronic AML1 P-UTR (T7-MAP) and AML1 D-UTR (T7-MAD), respectively (Fig. 3A). In vitro translation reactions were performed with RRL. Two types of assays were done, in vitro translation in the presence of [35S]methionine (Fig. 3B) and translation followed by a CAT activity assay (Fig. 3C). The results demonstrated that D-UTR mediates translation of the downstream cistron with an efficiency similar to that of the control construct containing the authentic CAT 5′UTR (Fig. 3B and C, lanes a and c); however, its translation-promoting activity was orientation dependent (lane b). The P-UTR, on the other hand, caused marked inhibition of translation of the downstream CAT relative to control (lanes c and e). These results indicate that in vitro, the template activity of P-UTR-CAT is markedly lower than that of the D-UTR-CAT construct and support the thesis that the P-UTR has the potential to negatively regulate the translation of AML1 mRNA.

**Picornavirus 2A protease abrogates D-UTR- and not P-UTR-mediated translation.** We next questioned whether the P- and D-UTRs are capable of mediating cap-dependent translation. Monocistronic capped mRNAs of T7-MAP and T7-MAD (Fig. 4), as well as of M-CAT and M-EMCV, which contained the highly active IRES of EMCV (7, 19), were made. The four mRNAs were translated in vitro in the presence of increasing amounts of 2A protease (Fig. 4). The picornavirus 2A protease cleaves the initiation factor eIF-4G, causing inhibition of cap-dependent translation of cellular mRNA while promoting the IRES-dependent translation of viral mRNA (59). Translation of the T7-MAD transcript, containing the D-UTR, was inhibited by the 2A protease in a dose-dependent manner. Similar inhibition was observed with control M-CAT mRNA, which contained the authentic CAT 5′UTR. On the other hand, translation of the T7-MAP mRNA, which contained the P-UTR, was not affected by the addition of 2A protease, and translation mediated by the IRES-containing
EMCV 5'UTR was only slightly reduced. The latter may reflect the involvement of eIF-4G in EMCV 5'UTR-mediated translation initiation (48). Taken together, the data strongly suggest that the P-UTR directs translation via a cap-independent mechanism and raised the possibility that it contains an IRES. The P-UTR contains IRES characteristic motifs and directs IRES-dependent translation in a bicistronic construct. All in all, there are no sequence similarities among IRES regions of viral and cellular origins (27). However, they have common structural motifs, a few of which could be detected in AML1 P-UTR. These included an oligopyrimidine tract located at the 3' part of the 5'UTR and a short sequence, UUUCC, which is complementary to the 3' end of the 18S rRNA. The latter is considered a functional analog of the known Shine-Delgarno sequence (26, 49). A Y-shaped stem-loop structure upstream of the AUG codon is an additional motif common to many viral and cellular IRESees (1, 4, 26, 27). A typical hairpin structure was found in these IRESees between the Y-shaped structure and the initiator AUG (27). Using a computerized RNA-folding program (28), the overall secondary structure of the P-UTR was deduced. It folds into a highly stable array of stem-loop structures which will most probably inhibit translation via the ribosome-scanning mechanism (results not shown). The program predicted that AML1 P-UTR folds into an array of hairpins and Y-shaped structures which are spaced along

FIG. 2. Expression pattern of AML1 mRNAs and the corresponding protein products. (A) Profiles of AML1 mRNAs in K562 and Daudi cell lines are shown in the left panel. Northern blots of poly(A)⁺ mRNA from K562 and Daudi cells were probed with runt domain (SmaI fragment encompassing nucleotides 1745 to 2135 of AML1a cDNA [29]). Of note, the AML2 gene, a member of the runt domain gene family, which is carried on chromosome 1p36, is highly expressed in Daudi cells but not at all in K562 cells (31). Profiles of AML1 proteins in K562 and Daudi cells are shown in the right panel. Proteins extracted from the cell lines were subjected to immunoblotting with the α-TD or α-distal antibodies described in the legend to Fig. 1B. (B) Profiles of AML1 mRNAs and proteins in lymphocytes, thymocytes, and HUVEC. Poly(A)⁺ mRNA and proteins were extracted and analyzed as in panel A. (C) Expression of AML1 mRNAs and proteins in lymphocytes freshly isolated from blood samples and after a 48-h incubation with or without TPA (1.5 ng/ml) and ConA (3.6 mg/ml). Following hybridization, the blot was stripped and rehybridized with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Films were scanned using a UMAX PowerLOOK II scanner, and band densities were determined by the Image Gauge V3.0 program. With nontreated lymphocytes (lanes d and g) as a reference (i.e., 1.0), lanes e and f gave densities of 1.98 and 1.52, respectively, and lanes h and i gave densities of 1.46 and 1.12, respectively. The probe used in panels B and C was a 2.5-kd SalI fragment encompassing nucleotides 1 to 2500 of AML1a (29). Due to the relative stability of AML1 mRNAs (29), the reduction seen following TPA and ConA treatment in panel C, lanes h and i, is very small. The slower-migrating protein bands in lanes h and i reflect a nonspecific reaction of the α-distal antibodies.
the UTR. Of note, a region of ~100 nucleotides immediately upstream of the initiator AUG, which is highly conserved in the mouse homologue of AML1, the CBFA2/RUNX1 mRNA, can form the characteristic Y-shaped stem-loop structures mentioned above (data not shown).

Bicistronic constructs have been previously used to identify both viral and cellular IRESs (reviewed in reference 18). To investigate the possibility that the P-UTR possesses an IRES activity, it was cloned into the bicistronic vector BAP (bicistronic AML1 P-UTR), in which transcription is mediated by the T7 promoter or the highly active promoter-enhancer region of cytomegalovirus and the first cistron CAT is followed by the P-UTR linked to LUC (Fig. 5A). Transcription generates capped transcripts that direct cap-dependent translation of CAT, whereas LUC is produced only when the intercistronic P-UTR confers an IRES-dependent translation. BAP directed an efficient translation of LUC in the RRL system (Fig. 5B), comparable to other cellular IRESs that confer translation in

FIG. 3. In vitro translation assays of T7-UTR-CAT constructs. (A) Schematic representation of the monocistronic constructs. Plasmids T7-UTR-CAT contain the D-UTR (T7-MAD) in the correct and reverse orientation, the P-UTR (T7-MAP) in both orientations, and the control M-CAT with the authentic CAT 5' UTR. Arrowheads represent the T7 promoter, boxes represent the CAT coding region, and arrows represent 5' UTRs. (B) In vitro translation assays. mRNAs (0.5 μM) were generated as described in Materials and Methods, using the plasmids shown in panel A, and translated in RRL in the presence of [35S]methionine. Labeled proteins were analyzed by PAGE, followed by autoradiography. Dried gels were exposed to a phosphorimaging plate, and radioactivity was recorded with a phosphorimager (FUJIX BAS 2500) using the Image Gauge V3.0 program. (C) Activity assay of in vitro translation products. CAT activity was measured in nonradioactive reactions by the phase extraction assay as described in Materials and Methods. The values correspond to arbitrary units given by the scintillation counter. Bars represent the mean and standard error for three independent experiments.

FIG. 4. Differential effect of picornavirus 2A protease on translation mediated by different 5'UTRs. A plasmid containing the 2A coding region downstream of the EMCV IRES was translated in vitro in a separate tube. RRL translation assay mixtures were preincubated for 30 min at 37°C with increasing amounts of the in vitro-made 2A protease. At this time point, mRNAs generated as in the experiment in Fig. 3, using the plasmids M-CAT, T7-MAP, T7-MAD, and M-EMCV, were added and reaction mixtures were incubated for an additional 1 h (see Materials and Methods). CAT activity was measured as indicated in the legend to Fig. 3. Values represent arbitrary units given by the scintillation counter. Numbers inside rectangles are relative CAT activities out of 100% attained in assays with no 2A protease. Bars represent the mean and standard error for three independent experiments.

FIG. 5. Translation of bicistronic CAT and LUC vectors in RRL. (A) Schematic representation of the bicistronic constructs. BAP contained the proximal 5'UTR, pCPL contained the PDGF/c-sis 5'UTR, and BiEMC contained the EMCV 5'UTR. (B) Equimolar capped bicistronic mRNAs were translated in RRL, and CAT and LUC activities were measured as described in Materials and Methods. Data are presented as arbitrary CAT and LUC values. Bars represent the mean and standard error for two independent experiments.
RRL (22, 42, 43, 67). In comparison, the P-UTR IRES was as active as the EMCV IRES (Fig. 5B) and much more active than the PDGF/c-sis IRES, which is known to perform poorly in RRL (4, 56). To further characterize the IRES activity of P-UTR and to rule out the possibility that the BAP-directed translation of LUC was due to a reinitiation past the CAT cistron, two new bicistronic plasmids were constructed (Fig. 6A): BhAP, in which a hairpin structure ($\Delta G = -40$ kcal/mol) was inserted in BAP upstream of the CAT cistron, and BAP-Rev, in which a fragment bearing the most 3′ 600 bp of P-UTR was inverted. BAP and BhAP were used to program RRL, and the resulting products were analyzed by enzymatic assays of CAT and LUC (Fig. 6B). Constructs were also transfected into K562 cells, a hematopoietic cell line expressing AML1 (29) (Fig. 6C). Comparison of BAP and BhAP showed that CAT production was strongly inhibited by the hairpin structure whereas P-UTR-directed LUC production was hardly affected (Fig. 6B to D). These opposite effects of the hairpin on the activities of the CAT and LUC cistrons in BhAP indicated that the two cistrons were independently translated and excluded the possibility of reinitiation past the CAT region. Consistent with this, modification of the P-UTR in BAP-Rev significantly reduced LUC activity in transfected K562 cells (Fig. 6E).

The observed LUC activity in K562 cells could have originated from truncated monocistronic templates that were produced through cleavage of the original transcript or by the use of a cryptic promoter within the UTR. To examine the intactness of the RNA, mRNA from transfected K562 cells was extracted and analyzed by Northern blotting with a 590-bp probe from the 5′ region of the LUC coding region. Prominent mRNA bands of the expected sizes (5.1 and 4.0 kb, respectively) were detected in poly(A)$^+$ mRNAs extracted from cells transfected with BAP and BiEMC (Fig. 6F). As a control, poly(A)$^+$ mRNA from nontransfected cells was analyzed and showed no specific hybridization to the LUC probe. Taken together, the results for LUC production by bicistronic BAP, translation inhibition by the hairpin, reduced LUC production by BAP-Rev, and the mRNAs analysis demonstrate that CAT and LUC production occurred through translation of the bicistronic templates and indicate that the P-UTR contains an IRES activity.

The P-UTR IRES displays differential activities in various cell lines. As indicated above, the presence of two 5′UTRs in AML1 mRNAs, one of which has a highly structured IRES, raised the possibility that like the viral IRES (8), the P-UTR is involved in the cell-specific transcription encoded in Fig. 2. To further address this issue, BAP-directed LUC activity was assayed in several cell lines. The repertoire included two hematopoietic cell lines that express high levels of the AML1 P-UTR-bearing mRNAs, i.e., K562 (early myeloblast/erythroblast) and Jurkat (T cells) and three nonhematopoietic cell lines, 293 (embryonic kidney cells), HeLa (epithelial), and SV80 (fibroblasts), which express very low levels, if any, of AML1. Each cell line was independently transfected with each of the three plasmids pCL, BAP, and BiEMC (Fig. 7A). In each case, the CAT activity served as a direct measure of the overall transfection-transcription efficiency while the LUC/CAT ratio monitored IRES activity. In addition, the performance of the P-UTR IRES in the transfected cells was compared with that of EMCV IRES, which was previously shown to be active in a variety of cell lines (8). The results showed that the P-UTR IRES displayed cell specific activity (Fig. 7B). In the nonhematopoietic cells (293, HeLa, and SV80) and in vitro, the BAP-directed LUC activity was significantly lower (low LUC/CAT ratio) than that of the control pCL plasmid or BiEMC (Fig. 7B). On the other hand, in Jurkat and K562 cells, 2302 POZNER ET AL. MOL. CELL. BIOL.
the BAP IRES not only was more active than that of pCL but also was even more active than that of BiEMC (Fig. 7B). We conclude that the P-UTR IRES functions best in cells that normally express AML1 mRNAs bearing the P-UTR. This conclusion is supported by the observation that the AML1 IRES activity was also low in hematopoietic B cells, such as Bjab and SKW6.4, that do not endogenously express the P-UTR mRNAs, compared to the T-cell lines Jurkat and SUP-T1 (Fig. 7C).

AML1 IRES activity is modulated during differentiation of K562 cells. (i) The activity of P-UTR is enhanced upon megakaryocytic differentiation. As shown above, K562 cells express relatively high levels of AML1 mRNAs, the majority of which are the P-UTR-bearing species (Fig. 2A). Despite the high mRNA level, the level of AML1 proteins was low (Fig. 2A). This was attributed to the relatively low translatability of the P-UTR-containing mRNAs (Fig. 2 and 3). K562 cells display both megakaryocytic and erythrocytic antigens and can be induced to differentiate into either lineage (52). We questioned whether during these differentiation processes alterations occur in the IRES-dependent translation of AML1 mRNAs. To address this issue, cells were treated with TPA, which induces K562 differentiation toward the megakaryocytic lineage (64); the cells became large and contained multiple separated nuclei that occasionally were lobulated and irregularly shaped. The IRES activity of P-UTR was assayed in TPA-treated and untreated cells. Each of the four plasmids shown in Fig. 8A was transfected into cells, and the LUC/CAT ratio was used as a measure of IRES activity. Results for each individual experiment were normalized to values obtained with pCL. Megakaryocytic differentiation of K562 cells was associated with a 2.5-fold increase in the IRES activity of P-UTR (Fig. 8B). This value is comparable to that reported for the differentiation-linked IRES activity of PDGF/c-sis mRNA (4). The activity of the positive control IRES (BiEMC) was raised fivefold. Significantly, none of the tested plasmids showed increased activity in TPA-treated 293 cells (Fig. 8B).

To correlate the above results with the endogenous situation, changes in the profile of AML1 mRNAs and proteins during TPA-induced differentiation were examined. As noted above, the prominent AML1 mRNAs in K562 are the P-UTR-bearing 8- and 4-kb species. The size difference of these mRNAs is mainly due to their different 3'UTRs (29). Upon TPA treatment, an increase in the amount of these mRNA species was noticed after 45 h (Fig. 8C). Western blot analysis of proteins extracted from TPA-treated cell cultures showed that the level of the 53-kDa AML1 protein started to increase earlier, at 10 to 20 h after TPA addition, and continued through the 45-h time point (Fig. 8C). Considering that this AML1 protein product did not react with the α-distal antibodies, this suggests that it was translated from a P-UTR-containing mRNA. Taken together, the results of the transfection assays and the in vivo studies indicate that megakaryocytic differentiation of K562 involves an increase in IRES-dependent translation of AML1 mRNA.

(ii) Erythroid differentiation of K562 abrogates the IRES activity of P-UTR. We next assessed the changes in IRES activity during differentiation of K562 into erythrocytes (53). In this study, we used sodium n-butyrate (NaB) and arabinosylcytosine (Ara-C), two reagents that stimulate K562 differentiation toward the erythroid lineage. When applied to cells, they both induced hemoglobinization in a time-dependent manner...
(Fig. 9A). IRES activity in differentiated K562 cells was monitored and normalized to the pCL standard, as mentioned above (Fig. 8). Induction of erythroid differentiation resulted in reduced activity of BAP (Fig. 9B). Ara-C, the more potent differentiation inducer (Fig. 9A), caused 50% reduction in the IRES activity of P-UTR, as well as that of BiEMC, while NaB, the less effective reagent, reduced BAP activity by 25% and increased BiEMC activity (Fig. 9B). No consistent, quantifiable changes in IRES activity were noted in 293 cells treated with either Ara-C or NaB (Fig. 9C). The data show that in contrast to megakaryocytic differentiation of K562, erythroid differentiation was associated with a diminished IRES-dependent translation of AML1 mRNAs. All in all, the above results indicate that translational regulation through an IRES-dependent activity of the P-UTR plays a role in AML1 expression during differentiation of K562 cells and suggest the involvement of AML1 5’UTRs in the regulated expression of the gene during hematopoiesis.

DISCUSSION

The runt domain transcription factors AML/RUNX are key regulators of gene expression in diverse biological processes such as hematopoiesis and osteogenesis (17, 36, 45, 51, 61). The three runt domain proteins AML1/RUNX1, AML2/RUNX3, and AML3/RUNX2 bind to the same DNA sequence (37); therefore, their pleiotropic functions must result from a regulated, temporally specific and tissue-specific expression of the genes (10, 29, 51, 57). However, surprisingly little is known about the molecular mechanisms regulating their temporally specific and cell type-specific expression. We have previously reported that transcription of AML1 is initiated at two promoter regions generating several mRNA species that differ at their 5’ leader exons (12, 29). In this study, we demonstrate that alternative promoter usage coupled to translation control are key features regulating expression of AML1.
shorter (452-bp) D-UTR directs efficient translation of the downstream ORF, whereas the longer (1,631-bp) P-UTR is much less active and supports tightly regulated translation. The low translational efficiency of P-UTR was attributed to its length and the cis-acting elements along it. The latter consist of several long GC-rich regions, which form highly stable secondary structures, and 15 cryptic uAUG codons, some of which are followed by short uORFs. These two elements were previously implicated in down regulation of translation (reference 35 and references therein).

The P-UTR mediates a cap-independent translation. It is not affected by the 2A protease and directs internal initiation and LUC translation from a bicistronic mRNA in the presence of an upstream hairpin structure. Activity of the P-UTR is also orientation dependent; translation of the internal LUC cistron is abolished by inversion of the proximal half of this 5'UTR. Based on the above data, we concluded that the P-UTR-bearing AML1 mRNAs belong to the growing group of cellular mRNAs with established 5'UTR-containing IRES. The D-UTR, on the other hand, directs cap-dependent initiation. It is highly active both in vivo and in the RRL, and its activity is inhibited by the 2A protease. When placed within the intercistronic region of the bicistronic construct, the D-UTR allowed some translation of the downstream LUC cistron; however, insertion of a hairpin structure in front of the first cistron diminished the translation of both CAT and LUC (data not shown). These data indicate that the D-UTR lacks IRES activity and emphasize the importance of using the hairpin-containing plasmids as controls.

Comparison of IRES regions of picornviruses and of several cellular mRNAs (Bip, FGF-2, and PDGF-2) revealed the existence of conserved Y-shaped elements (27, 56). Similar Y-shaped structures were identified in the P-UTR of AML1 and its mouse homologue in this study. Analysis of picornavirus IRES activity with respect to sequence and structure conservation, translation efficiency in RRL, and effect on activity by the 2A/L proteases classified these IRESes into three different groups (8). The AML1 initiator AUG codon is located immediately at the 3' end of the postulated Y-shaped structure. Furthermore, the P-UTR IRES was relatively active in RRL and was hardly affected by the 2A protease. It meets the criteria of type II IRES found in EMCV and foot-and-mouth disease virus (8). The IRESes of picornavirus and hepatitis C virus were also compared for their ability to support internal initiation in six different cell lines. IRES efficiency varied considerably from one cell type to another, presumably reflecting the presence or absence of IRES-specific factors (8). In this study we observed that in certain hematopoietic cell lines (K562 and Jurkat) the AML1 IRES was more active than that of EMCV while in nonhematopoietic cells (293, SV80, and HeLa) and in B cells (Bjab and SKW6.4) it was less active than the EMCV IRES. The lymphoblastoid T-cell line SUP-T1 supported a high IRES activity of both EMCV and P-UTR and may therefore contain IRES-specific factors needed for both of them. Assuming that IRES-specific proteins exist, one can speculate that their activity will vary in different cell types and in response to specific stimuli during the cell cycle and differentiation. As was previously shown for PDGF/c-sis (4), the AML1 IRES displays differentiation-dependent activity which conforms with the in vivo expression pattern of the gene. The molecular mechanisms underlying this differentiation-dependent IRES activity are not known. They may involve de novo synthesis of megakaryocytic IRES-promoting proteins or post-translational modifications of preexisting IRES regulatory proteins, which upon differentiation enhance the activity of the P-UTR IRES.

For picornviruses, the cap-independent, IRES-dependent translation grants an obvious advantage. The virus-encoded 2A protease destroys the cap-ribosome bridging factor eIF-4G, shuts off the cap-dependent translation of cell mRNA, and directs the cell machinery to translate the uncapped IRES-containing viral mRNA (59). The role of cap-independent translation in cell metabolism is less clear. Nevertheless, picornavirus IRES elements function in noninfected cells in the absence of any virus-encoded proteins (46), and it was reported (13, 34) that cellular proteins were identified to specifically interact with IRES elements. These observations support the notion that IRES-mediated translation is part of the cell control mechanism as well. IRES-bearing mRNAs could be translated under conditions when the common cap-dependent translation is diminished, for example when the eIF-4F holenzyme complex is inactivated by heat shock (25), growth arrest (50), serum starvation and γ-irradiation (14), or conditions of hypoxia (66). In addition, cap-dependent translation is extremely inefficient during mitosis, due to the presence of underphosphorylated and therefore nonfunctional eIF-4E (6). Thus, IRES-dependent translation provides an important regulatory element in the cascade of cellular gene expression. The highly structured 5'UTR may also play a role in mRNA localization and stability and serve to discriminate among various mRNA isoforms (see the discussion of AML1 isoforms below).

In such cases, translation via the IRES can circumvent the translation inhibition imposed by the structured 5'UTRs while preserving their unique functional properties. A long structured 5'UTR with cryptic uAUGs is a common feature in the mRNAs of many growth-regulatory genes (those encoding transcription factors, growth factors, cytokines, and receptors) (23). It is believed that the presence of IRESes in these UTRs provides a means for fine-tuning of their translation in time and space and in response to relevant stimuli (4, 68). The findings reported here strongly suggest that the major function of the P-UTR IRES-dependent translation is to tightly regulate the translation of AML1 mRNAs. We show that expression of AML1 is controlled at both the transcriptional and translational levels by alternative promoters and by cap-dependent or -independent translation mechanisms. This provides the necessary flexibility for production of the relevant type of AML1 protein isoforms in the appropriate amounts, at the proper time, and in the correct cell type. As documented here, the D-promoter, which mediates production of mRNAs bearing the D-UTR, is active during defined time windows in specific cell types, such as thrombocytes and Daudi cells, that produce large amounts of AML1 proteins. The P-promoter, on the other hand, is more active in certain hematopoietic cell lines (early myeloid) and in HUVEC. Moreover, the P-UTR IRES-dependent translation is increased during megakaryocytic differentiation of K562 cells during a time window when only the P-promoter is active, further highlighting the coupling between transcription regulation and translation control. The observed modulation of AML1 expression during differentiation of K562 may be related to the more recent finding that haploinsufficiency of AML1 impairs megakaryopoiesis (66). IRES-dependent translation may provoke the use of alternative initiation sites and lead to translation from the same mRNA molecule of different proteins with distinct functions (2, 42, 67, 68). A related issue emerged from our previous observation that AML1 D-UTR- and P-UTR-containing mRNAs encode proteins that differ at their N termini (12, 29), further highlighting the significance of the P-UTR IRES. In addition, the large repertoire of AML1 transcripts include mRNA species encoding proteins that differ at their C termini as well. It is possible that the various N and C termini interact
with other proteins of the transcription machinery to regulate the expression of target genes that function at a certain differentiation stage or at a defined time point. IRES-dependent translation can provide an effective solution to the demand for such a fine modulation. We recently demonstrated that the isoform AML1-b with the C terminus VWRP interacts with the corepressors TLE and Gro (30), implying a putative function for AML1 in transcriptional repression. Notably, the almost identical isoform AML1-a, lacking VWRP, failed to interact with TLE and Gro or to repress AML1-mediated transcriptional repression (30). We also found that one of the short isoforms, AML1-d, but not the full-length isoform, AML1-b, is capable of suppressing the in vivo growth and differentiation of tumors derived from embryonic stem cells (3).

In summary, in this work we show that expression of AML1 is controlled in vivo at both the transcriptional and translational levels through the usage of alternative promoters coupled to translation control. Translation is mediated by both cap- and IRES-dependent mechanisms. The AML1 IRES displays differentiation-dependent activity which conforms with the expression pattern of the gene. We argue that this cascade provides the necessary flexibility for production of the relevant AML1 protein isoforms in the appropriate amounts, at the proper time, and in the correct cell type.

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REFERENCES


15. Huez, I., L. Creancier, S. Audigier, M. C. Gensac, A. C. Prats, and H. Prats. 1995. Two independent internal ribosome entry sites involved in trans-
51. Podolsky, J. 2000. IRES-DEPENDENT TRANSLATION CONTROL OF AML1 EXPRESSION 2307

References