The K562 leukemia cell line is bipotential for erythroid and megakaryoblastic differentiation. The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) activates a genetic program of gene expression in these cells leading to their differentiation into megakaryoblasts, a platelet precursor. Thus, K562 cells offer a means to examine early changes in gene expression necessary for megakaryoblastic commitment and differentiation. An essential requirement for differentiation of many hematopoietic cell types is the down-regulation of c-myc expression, because its constitutive expression blocks differentiation. TPA-induced differentiation of K562 cells causes rapid down-regulation of c-myc expression, due in part to an mRNA decay rate that is 4-fold faster compared with dividing cells. A cell-free mRNA decay system reconstitutes TPA-induced destabilization of c-myc mRNA, but it requires at least two components for reconstitution. One component fractionates to the postribosomal supernatant from either untreated or treated cells. This component is sensitive to cycloheximide and micrococcal nuclease. The other component is polysome-associated and is induced or activated by TPA. Although in dividing cells c-myc mRNA decays via a sequential pathway involving removal of the poly(A) tract followed by degradation of the mRNA body, TPA activates a deadenylation-independent pathway. The cell-free mRNA decay system reconstitutes this alternate decay pathway as well.

Specific and timely changes in gene expression are required for cellular differentiation and proper embryonic development (reviewed in Ref. 1). Although gene expression can be regulated at many levels, the rates at which individual mRNAs decay is a major factor contributing to steady-state mRNA levels (reviewed in Ref. 2). The degradation rates of individual mRNAs can vary by an order of magnitude or more. Additionally, these rates can vary as a consequence of differentiation or a particular stage of the cell cycle. Such variations apparently comprise part of the normal pleiotropic response to cellular proliferation and differentiation signals and usually involve only a subset of mRNAs.

One such developmental system for studies of gene expression is the human cell line K562 (3, 4). K562 cells are bipotential for erythroid and megakaryoblastic development. Treatment of K562 cells with the protein kinase C activator 12-O-tetradecanoylphorbol-13-acetate (TPA) leads to loss of erythroid properties and maturation into megakaryoblasts, the precursor to platelets (reviewed in Ref. 5). The maturation processes resemble those observed during normal platelet development and include the following: inhibition of cellular replication; increase in cell volume; loss of glycoporphin A, an erythroid membrane protein; and synthesis of megakaryoblast proteins, such as glycoprotein IIIa, platelet peroxidase, thromboxane A2 receptors, the A and the B chains (c-sis) of PDGF, TGF-β1, urokinase-plasminogen activator and its specific inhibitor, type 1 plasminogen activator inhibitor.

Differentiation of a number of hematopoietic cell types appears to require inhibition of cellular proliferation. High level expression of the c-myc proto-oncogene appears to favor cellular proliferation over differentiation (reviewed in Ref. 6). This conclusion is based upon three observations: (i) c-myc expression declines an order of magnitude during the early stages of differentiation (7–9); (ii) continuous expression of a c-myc transgene linked to a strong promoter blocks differentiation (10–15); and (iii) oligonucleotides complementary to c-myc mRNA inhibit proliferation and induce differentiation (16). Thus, changes in the levels and timing of c-myc expression are essential to permit hematopoiesis, and it is thus important to understand at the molecular level how the cell effects these changes. Transcription of the c-myc gene appears to be constitutive during differentiation of K562 cells to megakaryoblasts (17). However, steady-state levels of c-myc mRNA decline at least 10-fold during the course of differentiation of murine erythroid leukemia cells, human monoblastic cells, and K562 cells (10–15, 17). This suggests that mRNA destabilization may exert a role in c-myc down-regulation during hematopoiesis. Here, I have examined the decay of c-myc mRNA during megakaryoblastic differentiation of K562 cells, utilizing both whole cells and in vitro mRNA decay assays. TPA induces a 20-fold decline in c-myc mRNA levels within 2 h. The decline likely results in part from a 4-fold acceleration in the decay rate of c-myc mRNA beginning 30–60 min after TPA addition. An in vitro mRNA decay system reconstitutes the destabilization effect and requires two cellular fractions: polysomes from TPA-treated cells and the 130,000 × g postribosomal supernatant (S130) from either untreated or TPA-treated cells. Therefore, TPA appears to activate or induce a polysome-associated com-
ponent that, in concert with the S130 factor(s), acts to promote the rapid decline in c-myc mRNA levels in differentiating megakaryoblasts.

In proliferating cells, c-myc mRNA rapidly decays by a sequential pathway involving rapid removal of the poly(A) tract to generate a deadenylated or oligoadenylated form; this is followed by degradation of the mRNA body generating 3′-terminal decay intermediates. Its decay then continues in a 3′ → 5′ direction (18). c-myc mRNA decays via this pathway in vitro as well (19, 20). However, in differentiating cells and in extracts of these cells, c-myc mRNA appears to decay, at least in part, by an additional mechanism that may not require prior conversion of polyadenylated molecules to a deadenylated form.

EXPERIMENTAL PROCEDURES

Restriction enzymes, RNAsin, and plasmid pGEM-Zf(+) were obtained from Promega Corp. (Madison, WI). Plasmid pSPM-1 was obtained from the American Type Culture Collection (Rockville, MD). TPA and RNase T1 were obtained from Sigma Chemical Co. (St. Louis, MO). RNase H and oligo(dT)-cellulose type 7 were from Amersham Pharmacia Biotech (Piscataway, NJ). Creatine phosphate, creatine phosphokinase, actinomycin D, and yeast tRNA were from Calbiochem (La Jolla, CA). Oligodeoxynucleotide synthesis was performed by Operon Technologies (Alameda, CA). All other reagents were molecular biology grade.

Plasmid Constructions and Radio-labeling of Probes—A radiolabeled probe for detection of human γ-globin mRNA by nuclease S1 mapping was prepared by 3′-end labeling of plasmid pDCY2 digested with EcoRI as described (19). The remaining probes, described below, were prepared by in vitro transcription of linearized plasmid templates using T7 RNA polymerase. A 245-nt, 32P-labeled, sense-strand RNA containing sequences (Alameda, CA). All other reagents were molecular biology grade.

Plasmid Constructions and Radiolabeling of Probes—A radiolabeled probe for detection of human γ-globin mRNA by nuclease S1 mapping was prepared by 3′-end labeling of plasmid pDCY2 digested with EcoRI as described (19). The remaining probes, described below, were prepared by in vitro transcription of linearized plasmid templates using T7 RNA polymerase and [α-32P]UTP (~800 Ci/mmol), unless otherwise noted, as described (19).

The same c-myc probe was used for both RNAse protection assays and for RNA blot analyses. It was prepared by in vitro transcription of Ssp1-digested plasmid pSP65myc(CLARI) (19). For PDGF-B (c-sis) mRNA, the 2.8-kb HindIII-Scl fragment of plasmid pSM-1 (21) was subcloned into the HindIII-Scl site of plasmid pGEM-Zf(+) to generate plasmid pGEM-huSIS-5′. The PDGF-B probe was prepared in vitro transcription of BamHI-digested plasmid pGEM-huSIS-5′. The probe protects a 277-nt region spanning nt 1949–2225. The β-actin probe was prepared by in vitro transcription of EcoRI-digested plasmid pGW02. This probe protects a 189-nt region spanning nt 1261–1449. For TGF-β1, cloned in GenScreen (GenBank® accession number AA148920) from the IMAGE consortium human cDNA library (Integrated Molecular Analysis of Genomes and their Expression (22)) was obtained from Genome Systems, Inc. (St. Louis, MO). A DNA fragment spanning nt 1607–1861 of the TGF-β1 cDNA sequence (23) was obtained by polymerase chain reaction using a 5′-primer incorporating a HindIII site and a 3′-primer incorporating an EcoRI site. The fragment was subcloned into the HindIII-EcoRI sites of pGEM7Zf(+). Primers 5′-primer + 1 to generate plasmid pTGFβ1 (nt 1607–1861). The TGF-β1 probe was prepared by in vitro transcription of HindIII-digested plasmid pTGβFβ (nt 1607–1861) using T7 RNA polymerase. A 245-nt, 32P-labeled, sense-strand RNA containing the c-myc coding region determinant (CRD (24)) was prepared by transcription of EcoRI-digested plasmid pGEM-mycXhoI(Nsi) (gift of J. Ross) using [α-32P]GTP. All probes were labeled to a specific activity of at least 20,000 cpm/μmol.

Actinomycin D Treatment of Cells, Preparation of RNA, and RNA Analyses—TPA was dissolved in 100% ethanol, and its concentration was determined by absorbance at 333 nm, using ε = 5400. Exponentially dividing K562 cells, a human cell line established from a patient with chronic myeloid leukemia in blast crisis (3, 4), were cultured for 1 h either without or with TPA (20 ng/ml final concentration). Cells were then cultured with 5 μg/ml actinomycin D for various lengths of time at 37 °C to inhibit transcription. For each time point, cells were harvested and total RNA was prepared by lysis of cells, phenol extraction of proteins, and pelleting of RNA through a pad of CsCl as described (25). RNA concentrations were determined spectrophotometrically by absorption at 260 nm. In some experiments 10-μg aliquots of RNA from each sample were separated into poly(A) + and poly(A)– by batch adsorption and elution with oligo(dT)-cellulose as described (18). γ-Globin mRNA was detected by S1 nuclease mapping as described (18). c-myc, PDGF-B, TGF-β1, and β-actin mRNAs were detected by RNase P1 + T1 protection assays as described (19). Bands were quantitated by capturing the gel images from x-ray film using a Kodak DC120 digital camera and then analyzing the images using 1D Image Analysis Software (version 3.0; Eastman Kodak, New Haven, CT).

RESULTS

TPA-induced Differentiation and Down-regulation of c-myc mRNA—TPA treatment of K562 cells activates a genetic program that results in changing patterns of gene expression leading to megakaryoblastic differentiation. c-myc mRNA is known to decline in K562 cells after 1 day of TPA treatment (17). However, I wished to determine if there were rapid fluctuations in c-myc levels at early times after induction of differentiation. To examine potential fluctuations in c-myc expression during differentiation, exponentially dividing K562 cells were cultured in the presence of TPA. RNA was extracted from cells at various times, and the level of c-myc mRNA was measured by RNase P1 + T1 protection assay employing a 620-nt radiolabeled RNA probe complementary to the 3′-terminal 210 nt of c-myc mRNA (Fig. 1). The time-zero RNA generated four protected fragments corresponding to c-myc mRNA molecules polyadenylated at four closely spaced sites. Historically, these are referred to as poly(A) site 2 (19). Quantitation of band intensities as a function of time indicated that the level of c-myc mRNA declined about 20-fold within 2 h. This effect was also
reproducible, having been observed in two independent experiments and with multiple repeats of the RNase protection assay (data not shown). After 2 h, levels of c-myc mRNA then increased transiently and then decreased again over the next several days. Because K562 cells can lose their capacity for differentiation during long-term culture (33), the levels of \( \gamma \)-globin, PDGF-B, TGF-\( \beta \)1, and \( \beta \)-actin mRNAs were also examined to ensure that the cells have maintained their fidelity for differentiation. Both PDGF-B and TGF-\( \beta \)1 are markers for megakaryoblastic differentiation (5). The levels of \( \gamma \)-globin mRNA, an erythroid-specific mRNA, remained relatively constant for 24 h but then declined about 80% by 72 h. This is consistent with the loss of erythroid properties. By contrast, the levels of both PDGF-B and TGF-\( \beta \)1 mRNAs were induced by TPA. \( \beta \)-Actin mRNA levels were maximal at 6 h of TPA treatment and then steadily declined between 6 and 72 h. This result is consistent with other reports concerning TPA-induced differentiation of K562 cells to megakaryoblasts, where a decrease in \( \beta \)-actin expression was reported to occur as differentiation proceeded (34). Taken together, these results are consistent with TPA-induced maturation of K562 cells to megakaryoblasts.

Rapid down-regulation of c-myc mRNA levels is an essential step in the early stages of differentiation of a number of hematopoietic cell lineages (10–15). In K562 cells, the levels of c-myc mRNA decline (Fig. 1) even though transcription of the gene remains active (17). To determine if c-myc mRNA is destabilized during this early period, K562 cells were treated with TPA for 1 h and exposed to actinomycin D to inhibit transcription. Cells were harvested for purification of total RNA at various time points. Decay of c-myc mRNA was examined by RNase protection assay. The mRNA was degraded 4-fold faster in TPA-treated cells (estimated half-life 10 min) than in untreated cells (half-life 45 min) (Fig. 2A, compare lanes 10–16 with lanes 3–9; Fig. 2B; see also Fig. 6). By contrast, \( \gamma \)-globin mRNA remained stable (Fig. 2C). Thus, mRNA destabilization is selective during the early stages of differentiation, and destabilization of c-myc mRNA likely contributes to its rapid decline during TPA treatment.

Reconstitution of TPA-induced mRNA Destabilization by Cell-free Extracts—Our long-term goal is to define the cellular components required for regulation of c-myc mRNA decay in dividing and differentiating cells. We and others have utilized cell-free systems to dissect mRNA decay (reviewed in Ref. 35; see also Ref. 36). Polysomes, with or without the S130 fraction, are incubated for various times in a buffer containing monova-
c-myc mRNA Decay during Differentiation

Fig. 2. Comparison of c-myc mRNA decay in dividing and differentiating K562 cells. A, exponentially dividing K562 cells were either left untreated (control), or cultured with 20 ng/ml TPA for 1 h, or cultured sequentially first with 100 μg/ml cycloheximide (chx) for 1 h and then with 20 ng/ml TPA for 1 h. Actinomycin D was added to each flask. At various times thereafter, cells were harvested and total RNA was prepared. c-myc mRNA was analyzed for each time point by an RNase P1+T1 protection assay using a 620-nt, 32P-labeled probe spanning the last 210 nt of c-myc mRNA as described under “Experimental Procedures.” Protected fragments were separated in a 5% polyacrylamide/7 M urea gel and detected by autoradiography. pA2 indicates the protected fragments corresponding to poly(A) site 2 molecules (19). Lane 1 is an analysis of 15 μg of total RNA from exponentially dividing cells to demonstrate probe excess. Lane 2 contains 15 μg of E. coli tRNA as a negative control. Lanes 3–9 are RNAs from control K562 cells. Lanes 10–16 are RNAs from TPA-treated cells. Lanes 17–20 are RNAs from cells sequentially treated with cycloheximide then TPA. Band quantitation of the RNase protection assay. Bands were quantified by analysis of a digitally captured video image of the gels in A and are plotted as a percentage of c-myc mRNA remaining at each time point compared with time zero. C, analysis of γ-globin mRNA decay in K562 cells. 0.5 μg of the indicated RNA samples from A were assayed for γ-globin mRNA by S1 nuclease mapping using a 32P-labeled probe that spans the last 167 nt of γ-globin mRNA. Protected fragments were detected by autoradiography. Lane 1 is an analysis of 15 μg of total RNA from exponentially dividing cells to demonstrate probe excess. Lane 2 contains 15 μg of E. coli tRNA as a negative control. Lanes 3–6 are RNA from control cells. Lanes 7–10 are RNA from TPA-treated cells.

 lent and divalent cations, ATP/GTP, an ATP-regenerating system, and RNasin (to inhibit indiscriminate degradation of all RNAs). The decay of individual mRNAs is then assessed by nuclease protection assays.

Trans-acting factors that contribute to the decay of c-myc mRNA are localized in both the polysome and S130 fractions (19, 20, 28). Thus, to identify cellular factors required to reconstitute TPA-induced destabilization of c-myc mRNA in vitro, polysome and S130 fractions from untreated (control) and TPA-treated cells were mixed in a variety of combinations and incubated for various times in decay reactions. Decay of endogenous, polysome-bound c-myc mRNA was then assessed in each case by RNase protection assays. Either control S130 or TPA S130 mixed with control polysomes accelerated c-myc mRNA decay relative to reactions supplemented with BSA (Fig. 1A, compare lanes 8–16 with lanes 3–7), consistent with earlier identification of an S130-associated destabilizing activity in dividing cells (28). However, control S130 and TPA S130 were equally effective. Likewise, either control S130 or TPA S130 mixed with TPA polysomes accelerated c-myc mRNA decay relative to reactions supplemented with BSA (Fig. 3B, compare lanes 8–16 with lanes 3–7). Again, control S130 and TPA S130 were equally effective. However, the magnitude of the destabilization effect was greatest in reactions containing polysomes from TPA-treated cells and S130 from either control cells or TPA-treated cells (compare Fig. 3B, lanes 8–16 with Fig. 3A, lanes 8–16). These data are depicted in graph form in Fig. 3C (left panel). Thus, a TPA-inducible component appeared to be associated with polysomes. This TPA-inducible, polysome-associated component(s) required the S130 fraction, however, because c-myc mRNA decay rates were comparable in polysome-containing reactions supplemented with BSA instead of the S130 (compare Fig. 3A, lanes 3–7 with Fig. 3B, lanes 3–7). Additionally, the TPA-induced destabilizing effect observed with the S130s and TPA polysomes was selective, because γ-globin mRNA was stable under these conditions (Fig. 3D). Taken together, the results indicate that: (i) TPA-induced destabilization of c-myc mRNA can be reconstituted in vitro; (ii) destabilization in vitro requires polysomes from TPA-treated cells and S130; and (iii) the S130 from either untreated or TPA-treated cells is equally effective. Thus, although the TPA-inducible component(s) fractionates with polysomes, it must act in concert with S130 components to effect destabilization of c-myc mRNA.

Cycloheximide Blocks TPA-induced mRNA Destabilization—The in vitro decay experiments described above showed that the S130 from either untreated or TPA-treated cells was required for the TPA-induced destabilization effect. Prior work...
c-myc mRNA Decay during Differentiation
showed that an S130-associated destabilizing activity for c-myc mRNA could be inhibited by treatment of cells with the translational inhibitor cycloheximide (28). To determine if the S130 activity that participates in TPA-induced destabilization was also affected by cycloheximide, K562 cells were treated with 100 μg/ml cycloheximide for 2 h prior to a 1-h treatment with TPA. S130 was prepared for in vitro mRNA decay reactions. This S130 fraction was incubated in reactions containing polysomes from cells treated with TPA only and then analyzed for c-myc mRNA decay. c-myc mRNA was stabilized in these reactions compared with reactions containing S130 from cells not treated with cycloheximide (Fig. 3B, compare lanes 17–21 with lanes 8–16). The in vitro mRNA decay data for extracts of cycloheximide/TPA- and TPA-treated cells are depicted in graph form in Fig. 3C. The effect of cycloheximide was due solely to the S130 fraction, because the polysomes utilized in this experiment were prepared from cells treated with TPA only (i.e. without cycloheximide). The stabilization effect of cycloheximide occurred in vivo as well; culturing cells with cycloheximide prior to TPA treatment stabilized c-myc mRNA in whole cells (Fig. 2A, compare lanes 17–20 with lanes 10–16; see also Fig. 2B). Thus, a labile destabilizing activity that acts to promote c-myc mRNA decay in exponentially dividing cells may also be required for TPA-induced destabilization of the mRNA during differentiation. This was addressed further by examining selected biochemical properties of the S130-associated activity from TPA-treated cells.

Partial Characterization of the S130-associated Activity—The requirement for a labile, S130-associated activity for TPA-induced destabilization of c-myc mRNA suggested that it is related to a destabilizing activity previously identified in the S130 of dividing cells (28). The S130 factor in dividing cells accelerates decay of c-myc mRNA in vitro but does not affect other mRNAs, such as δ- or γ-globin, H4 histone, or total polysome-bound poly(A+), mRNA. It is also sensitive to cycloheximide treatment of cells, as shown above for the activity from TPA-treated cells. Moreover, it is inactivated by micrococcal nuclease treatment but not by proteinase K treatment, suggesting that it consists of a nucleic acid component(s) and proteinase-resistant protein(s) (28).

The S130-associated factor required for TPA-induced destabilization of c-myc mRNA possessed similar properties. Micrococcal nuclease treatment of S130 from TPA-treated cells inactivated the activity (Fig. 4, compare lane 5 with lane 3), but proteinase K did not (Fig. 4, compare lanes 8 and 9 with lane 3). Several control treatments indicated the following. (i) Inactivation of destabilizer activity required calcium, indicating that it was dependent on micrococcal nuclease (Fig. 4, compare lane 7 with lane 3). (ii) Calcium alone did not inactivate the destabilizer activity (Fig. 4, compare lane 4 with lane 3), which again supports the conclusion that inactivation was micrococcal nuclease-dependent. (iii) After micrococcal nuclease treatment, competitor RNA was added to the treated S130 prior to in vitro mRNA decay reactions. This control reaction was performed to eliminate the possibility of a masking artifact whereby micrococcal nuclease itself, or RNA-associated proteins released by nuclease treatment, might mask c-myc mRNA in decay reactions and block its degradation (see Ref. 28). Addition of tRNA after micrococcal nuclease treatment of the S130 did not restore mRNA-destabilizing activity to the S130 (Fig. 4, compare lane 6 with lane 3). This suggests that nucleolytic destruction of the S130-associated destabilizer activity, and not a masking artifact, was responsible for loss of c-myc mRNA decay activity. Taken together, these results suggest that the S130-associated activity required for destabilization of c-myc mRNA in differentiating cells may be the same as, or similar to, the activity found in dividing cells.

Differentiation May Activate an Additional mRNA Decay Pathway(s)—In both extracts of exponentially dividing K562 cells and in whole cells, c-myc mRNA decays via a sequential pathway involving conversion of polyadenylated molecules to poly(A)-deficient ones, which are then degraded 3′→5′ (18–20, 33341).
To examine the in vitro decay pathway under conditions of TPA-induced differentiation, cell-free mRNA decay reactions were performed with polysomes and S130 prepared from TPA-treated K562 cells. However, due to the rapidity of c-myc mRNA degradation at 37 °C (see Fig. 3B), reactions were instead incubated at 20 °C to slow the decay rate and allow examination of poly(A) tract lengths. RNA samples were subjected to oligonucleotide-directed RNase H cleavage of c-myc mRNA for subsequent Northern blot analysis (i.e. RNase H mapping). This procedure permits a higher resolution analysis of poly(A) tracts than that of traditional Northern blotting. The blot was hybridized with a 32P-labeled probe specific to the 3′-end of c-myc mRNA to visualize poly(A) tract lengths. Consistent with earlier results (18, 19), the time-zero RNA showed a broad band indicative of heterogeneous lengths of poly(A) tracts within the population of c-myc mRNA molecules at the time of cell lysis (Fig. 5A, lane 1). With increasing incubation times, the intensity of the broad band representing c-myc mRNA molecules declined, indicating degradation. However, the lengths of the poly(A) tracts within the population did not appear to decrease significantly (Fig. 5A, lanes 2–7), suggesting that degradation occurred in a deadenylation-independent fashion. This result contrasts with the well-characterized observation of time-dependent shortening of poly(A) tracts in extracts of exponentially dividing cells (18–20, 28, 35, 36). Taken together, these observations suggest that c-myc mRNA is degraded via an alternate pathway in extracts prepared from differentiating cells.

For comparison, the decay pathway of c-myc mRNA was examined in whole cells during TPA-induced differentiation. K562 cells were treated with TPA for 1 h. At short time points after addition of actinomycin D, cells were harvested for RNA isolation. RNA samples were analyzed by RNase H mapping of c-myc poly(A) tracts (Fig. 5B). To estimate deadenylation rates, poly(A) tract lengths were measured as a function of time. Because the population of c-myc mRNA molecules contained poly(A) tracts of heterogeneous lengths, the length of the longest poly(A) tract detectable at each time point between 0 and 20 min was measured. (The signals for the 30- and 60-min time points were too faint to provide reliable measurements.) This analysis revealed a deadenylation rate of about 3 nt/min. A similar analysis of RNA samples from exponentially dividing cells indicated a deadenylation rate of about 2 nt/min (e.g. see Fig. 1 in Ref. 18). This 50% difference in deadenylation rates is not sufficient to account for the 4-fold shorter half-life of c-myc mRNA in differentiating cells versus dividing cells (10 and 45 min, respectively) as determined from the data in Fig. 2. Together, these results suggest that, during megakaryoblastic differentiation, c-myc mRNA molecules are degraded in part through an additional, deadenylation-independent pathway.

To explore this possibility further, the decay rates of c-myc mRNA in both the poly(A+) and poly(A−) RNA fractions were compared using RNA samples from dividing and differentiating cells treated with actinomycin D for various times. The rationale for this approach is based upon the following three observations. (i) Dividing cells contain both a poly(A+) and a poly(A−) population of c-myc mRNA molecules (18, 37). (ii) Kinniburgh and colleagues (37) demonstrated that poly(A−) c-myc mRNA appears to decay with slower kinetics compared with the rapid kinetics of poly(A+) c-myc mRNA decay in exponentially dividing promyelocytes (HL-60 cells). They interpreted this result in terms of a precursor-product relationship whereby the poly(A+) precursor mRNA decayed to a poly(A−) product, which then subsequently decayed (i.e. a sequential decay pathway). Thus, the apparent difference in kinetics was due to the rapidly decaying pool of poly(A+) c-myc mRNA replenishing the pool of poly(A−) c-myc mRNA. The pool of poly(A−) mRNA subsequently decreased in abundance by de-
c-my c-mRNA Decay during Differentiation

Fig. 6. Analyses of poly(A\textsuperscript{+}) and poly(A\textsuperscript{−}) c-mRNA decay in dividing and differentiating K562 cells. Actinomycin D was added to cultures of either dividing cells or cells treated with 20 ng/ml TPA for 1 h. Total RNA was isolated at the indicated time points. An aliquot of RNA from each time point was separated into poly(A\textsuperscript{+})\textsuperscript{1} and poly(A\textsuperscript{−})\textsuperscript{2} fractions by batch adsorption and elution with oligo(dT)-cellulose as described under "Experimental Procedures." RNAs were analyzed for c-myc mRNA by RNase P1+T1 protection assay and visualized by exposure to x-ray film. A, analysis of poly(A\textsuperscript{+}) (lanes 1–7) and poly(A\textsuperscript{−}) (lanes 8–14) c-myc mRNA in dividing cells. In B: Upper panel, analysis of poly(A\textsuperscript{+}) c-myc mRNA in differentiating cells using the same RNA samples described in Fig. 5B. Lower panel, analysis of poly(A\textsuperscript{+}) c-myc mRNA in differentiating cells using the same RNA samples described in Fig. 5B. In each case bands were quantified by analysis of video-captured images of the gels. Data are plotted as a percentage of c-myc mRNA remaining versus time. The plots are presented to the right of each gel panel.

cay once the poly(A\textsuperscript{+}) precursor pool was nearly depleted. (iii) By contrast, in differentiating HL-60 cells, Kinniburgh and colleagues (38) found that both poly(A\textsuperscript{+}) and poly(A\textsuperscript{−}) forms of c-myc mRNA decay with similar kinetics. They interpreted this result to mean that polyadenylated c-myc mRNA decayed directly without its prior conversion to a deadenylated form in differentiating HL-60 cells. Thus, comparisons of mRNA decay rates in poly(A\textsuperscript{+}) and poly(A\textsuperscript{−}) fractions of dividing and differentiating K562 cells should permit determination of the decay pathways operative in each case.

For analysis of K562 cells, RNAs from dividing and differentiating cells, treated with actinomycin D for various times, were separated into poly(A\textsuperscript{+}) and poly(A\textsuperscript{−}) fractions by oligo(dT)-cellulose chromatography. c-myc mRNA was analyzed in each case by RNase protection assays. Poly(A\textsuperscript{+}) c-myc mRNA decayed with first order kinetics with a half-life of about 45 min (Fig. 6A, lanes 1–7 and right panel). By contrast, poly(A\textsuperscript{−}) c-myc mRNA levels increased transiently between 0 and 30 min and then declined with first order kinetics (Fig. 6A, lanes 8–14 and right panel). These time-dependent changes in the levels of the poly(A\textsuperscript{+}) and poly(A\textsuperscript{−}) forms would be expected for a sequential, two-step process in which the second step (degradation of deadenylated molecules) occurred at a rate slower than the first step (poly(A) shortening). Thus, the data in Fig. 6A are consistent with the previously reported sequential decay pathway involving conversion of the polyadenylated molecules to a poly(A)-deficient state followed by rapid degradation of the poly(A)-deficient molecules (18–20, 37). However, the kinetic profile of c-myc mRNA decay in differentiating cells differed in several ways from the kinetic profile observed in dividing cells. (i) Decay of poly(A\textsuperscript{+}) c-myc mRNA from TPA-treated cells followed first order decay kinetics with a half-life of 12 min (Fig. 6B, upper panels). This is about 4-fold faster than the decay rate in dividing cells. (ii) Poly(A\textsuperscript{−}) c-myc mRNA in differentiating cells decayed with kinetics similar to those for poly(A\textsuperscript{+}) mRNA, again with a half-life of about 12 min (Fig. 6B, lower panels). These results, taken together with the observation that deadenylation rates differ by only about 50% in dividing and differentiating cells, suggest that differentiation activates an additional decay pathway that permits direct degradation of polyadenylated mRNA molecules without their prior conversion to a poly(A)-deficient form. The in vitro mRNA decay experiments shown in Fig. 5A provide support for this additional pathway as well.

Activity of the c-myc CRD-binding Protein in Extracts—Much work to date suggests that the coding region of c-myc mRNA, and not the 3′-UTR, controls the level of the mRNA during differentiation (12, 39–42). One region of the open reading frame implicated in regulation is the CRD, which spans nt 1705–1886 (24, 43). The 70-kDa CRD-BP is thought to bind the CRD and protect the mRNA from endonucleolytic attack. In cell-free decay experiments, addition of RNA containing the CRD accelerates decay of poly(A)-bound c-myc mRNA presumably by acting as a competitor for binding by CRD-BP. The
in the S130 fractions (Fig. 7B, lower panel). Thus, it is not likely that changes in CRD-binding activity per se of CRD-BP can account for TPA-induced destabilization of c-myc mRNA. However, this result does not eliminate the possibility that CRD-BP regulates c-myc mRNA decay during TPA-induced differentiation (see “Discussion”).

DISCUSSION

It is now well known that the level of c-myc expression sets the balance between proliferation and differentiation (6). Down-regulation of c-myc expression appears to be necessary for differentiation and maturation of a number of hematopoietic cell types. The data presented here indicate that down-regulation of c-myc mRNA in differentiating K562 cells occurs in part through a 4-fold destabilization of the mRNA. The destabilization effect can be reconstituted in a cell-free mRNA decay system using polysomes from TPA-treated cells and S130 proteins from either control or TPA-treated cells. The S130-associated factor(s) is active regardless of whether cells are treated with TPA or not. However, it is not active in extracts of cells treated with cycloheximide prior to induction of differentiation, suggesting that continuous translation is required for its activity. In this regard, the S130-associated activity described here appears similar to the cycloheximide-sensitive, c-myc mRNA destabilizer previously identified in the S130 of exponentially dividing K562 cells (28). The observation that the aforementioned destabilizer from dividing cells and the S130-associated factor required for TPA-induced destabilization are both sensitive to micrococcal nuclease treatment (Ref. 28 and Fig. 4) suggests that the activities require a nucleic acid component and are likely related. In any event, future work will be required to purify and fully characterize the S130-associated factor(s).

The S130-associated factor apparently exerts its effect in concert with a polysome-associated component(s) that is somehow affected by TPA-induced differentiation. What might the polysome-associated factor be? Because the coding region of c-myc mRNA seems to regulate its differentiation-dependent down-regulation (12, 39, 41, 42), one likely candidate is the 70-kDa CRD-BP. CRD-BP binds c-myc mRNA within a 182-nucleotide region encoding a portion of the helix-loop-helix/leucine zipper of c-Myc (24, 44, 45). When induced to dissociate from the mRNA in cell-free mRNA decay reactions, c-myc mRNA is exposed to endonucleolytic attack (24). This results in the destabilization of an already unstable mRNA. However, the RNA-binding activity of CRD-BP was not detectably altered in extracts of TPA-treated cells compared with extracts of control cells (Fig. 7). Thus, it is not likely that TPA treatment alters the RNA-binding activity per se of CRD-BP. Nonetheless, this protein could still modulate TPA-induced destabilization. For example, TPA might induce CRD binding by a protein that could displace CRD-BP, permitting targeted endonucleolytic degradation. In any event, future work will be required to identify the polysome-associated, TPA-induced factor(s).

Data presented here suggest that TPA-induced destabilization of c-myc mRNA involves, in part, a pathway that may be independent of deadenylation, both in vitro and in vivo (Figs. 5 and 6). Cell-based experiments of Swartwout and Kinniburgh (38) also suggested that differentiation of promyelocytic leukemia cells leads to activation of deadenylation-independent decay of c-myc mRNA. However, attempts to identify stable products of endonucleolytic cleavage have not been successful (data not shown). Nonetheless, there are several ribonucleolytic activities that could contribute to degradation of c-myc mRNA in a deadenylation-independent fashion in differentiating K562 cells. These include a polysome-associated endoribonuclease purified by Ross and colleagues (46) and the phosphorylation-
dependent RNase activity of the GAP-SH3 binding protein, G3BP (47). Both of these RNases can utilize c-myc mRNA as a substrate (46, 47). Alternatively, destabilization could involve deadenylation-independent decapping and 5'→3' degradation of c-myc mRNA via an Xrn1-like activity (48). Such a mechanism is responsible for nonsense-mediated mRNA decay in the yeast Saccharomyces cerevisiae (reviewed in Refs. 49–51) and possibly in mammalian cells as well (52).

In summary, c-myc mRNA is destabilized during differentiation of K562 cells to megakaryoblasts. A cell-free mRNA decay system reconstitutes this effect whereby the TPA-inducible component(s) fractionate(s) with polysomes, but it requires one or more constitutive, S130-associated factors acting in concert. Differentiation also may activate a decay pathway that does not involve deadenylation prior to degradation of the body of the mRNA; this alternate pathway appears to be operative not involve deadenylation prior to degradation of the mRNA; this alternate pathway appears to be operative. Future studies will be required to elucidate the details of the TPA-induced mRNA decay pathway and to identify the relevant trans-acting factors. The observation that destabilization of c-myc mRNA is reconstituted in vitro will aid both of these endeavors.

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