

Expression of the Transcription Factor, Spi-1 (PU.1), in Differentiating Murine Erythroleukemia Cells Is Regulated Post-transcriptionally

EVIDENCE FOR DIFFERENTIAL STABILITY OF TRANSCRIPTION FACTOR mRNAs FOLLOWING INDUCER EXPOSURE*

(Received for publication, July 13, 1995, and in revised form, November 9, 1995)

Jack O. Hensold^{‡§¶}, Carl A. Stratton[§], Diane Barth[‡], and Deborah L. Galson[¶]

From the [‡]University/Ireland Cancer Center, Department of Medicine and Case Western Reserve University, Cleveland, Ohio 44106, the [§]Department of Veteran's Affairs Medical Center, Cleveland, Ohio 44106, and the [¶]Arthritis Unit, Massachusetts General Hospital, Charlestown, Massachusetts 02129

Increased expression of the transcription factor Spi-1 (PU.1) results from retroviral insertion in nearly all Friend spleen focus-forming virus-transformed murine erythroleukemia cell lines and exposure of these cells to Me₂SO, induces their differentiation and decreases Spi-1 mRNA level by 4–5-fold. While these results suggest that alterations in Spi-1 expression have significant effects on erythroblast growth and differentiation, neither the cause nor the effect of the decrease in Spi-1 expression that follows Me₂SO exposure has been established. The experiments described here demonstrate that the effect of inducers on Spi-1 expression is regulated post-transcriptionally. Nuclear run-off transcriptions demonstrated that Spi-1 transcription was not decreased following Me₂SO exposure. Additionally, expression of a recombinant Spi-1 mRNA under transcriptional control of a constitutively active Rous sarcoma virus promoter was regulated identically to endogenous Spi-1 mRNA. The ability of Me₂SO to destabilize Spi-1 mRNA was selective, as the stability of the erythroid transcription factors GATA-1 and NF-E2 were not similarly effected. The effect of Me₂SO on the stability of Spi-1 mRNA provides a novel means of altering gene expression in these cells and is likely to have significance for the differentiation of these cells.

SFFV¹-transformed MEL cells are an established system for studying erythroid growth and differentiation. These cells are derived from mice infected with the Friend retrovirus complex, which includes the defective SFFV and a helper Friend murine leukemia virus (1). Three discrete molecular events have been identified, which contribute to the transformation of these erythroblasts. An early, proliferative phase of the disease results from interaction of the mutant *env* gene product of the SFFV with the erythropoietin receptor, producing ligand-inde-

pendent receptor activation (2). These infected erythroblasts are still able to differentiate and withdraw from the cell cycle. The subsequent acute erythroleukemic phase of the disease is associated with accumulation of undifferentiated erythroblasts. For essentially all of these erythroleukemias, genetic changes can be demonstrated in two additional loci. These include mutations or deletions of p53 (3, 4) and retroviral insertion and transcriptional activation of the Spi-1 (PU.1) gene (5, 6), which encodes a transcription factor (7, 8). How these changes interact to transform these cells remains unknown.

Spi-1 encodes a protein related to the *ets* family of transcription factors (9) which has a demonstrated role in regulating gene expression in monocytes and B-lymphocytes (10–12). Additional evidence suggests this transcription factor also plays a role in erythroid differentiation. Spi-1 is normally expressed in erythroid progenitors (CFU-E) (13), and mice lacking this gene die *in utero*, displaying a general defect in hematopoiesis including defective maturation of developing erythroblasts (14). In the erythroleukemic stage of Friend SFFV disease, overexpression of Spi-1 is a universal occurrence (6), and overexpression of Spi-1 has also been shown to immortalize erythroblasts in long-term bone marrow cultures (15). In addition, two related *ets* family genes, *fli-1* and *v-ets*, have also been implicated in erythroleukemic transformation (16, 17). These findings strongly suggest that increased expression of Spi-1 is significant for erythroblastic transformation and for blocking the normal differentiation of these cells.

MEL cells undergo terminal erythroid differentiation when exposed to Me₂SO or a number of other inducing agents (18), and the expression of Spi-1 decreases 4–5-fold in Me₂SO-exposed cells (13, 19). To understand how inducers cause differentiation of these cells, we investigated the regulation of Spi-1 expression in inducer-exposed cells. The data presented here demonstrate that transcription of Spi-1 is unaltered by Me₂SO exposure, suggesting that the stability of this mRNA is decreased by inducer exposure. A Spi-1 cDNA transcribed by a constitutively active RSV promoter is regulated identically to endogenous Spi-1, confirming that expression of this mRNA is regulated post-transcriptionally and confining the required sequences for this regulation to the coding region plus 10 nucleotides of 5'- and 3'-UT sequences. In contrast, the stability of mRNAs encoding the erythroid transcription factors, GATA-1 and NF-E2, are unaffected by inducer exposure. These findings demonstrate that inducers can alter gene expression via post-transcriptional mechanisms. The ability of Me₂SO to differentially affect transcription factor stability suggests that this

* This work was supported by Grant DK43414 from the National Institutes of Health and by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: UCRC 2, Suite 200, 11001 Cedar Ave., Cleveland, OH 44106. Tel.: 216-844-8245; Fax: 216-844-8230.

¹ The abbreviations used are: SFFV, spleen focus-forming virus; MEL, murine erythroleukemia; RSV, Rous sarcoma virus; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; UT, untranslated.

mechanism plays a significant role in altering gene expression in cells exposed to this agent.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture supplies were from Life Technologies, Inc., and fetal bovine serum was from Intergen (Purchase, NY). Me_2SO was from Eastman Kodak Co., and 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole was obtained from Calbiochem. Biochemicals were from Sigma. Enzymes and reagents for molecular biology were from Boehringer Mannheim, New England Biolabs, Pharmacia Biotech Inc., and Promega. Radionucleotides were from DuPont NEN. Membranes for blotting were from Schleicher & Schuell.

Cell Culture—MEL cells were subclones 745-PC4-B1-2A17 m¹ and -2A17 m³. These cells were derived from the original 745 cell line of Charlotte Friend and have been repeatedly subcloned to maintain high rates of differentiation when exposed to Me_2SO . The cells were grown in Dulbecco's modified Eagle's medium supplemented with 12% fetal bovine serum and 2 mM L-glutamine and maintained at concentrations that ensured logarithmic phase of growth ($0.5\text{--}10 \times 10^5$ cells/ml). Differentiation was induced by addition of 1.5% (v/v) Me_2SO to the growth medium and assessed by accumulation of β -globin mRNA.

Determination of *Spi-1* mRNA Level—Cells were grown with or without Me_2SO for the times described in the text. Cytoplasmic RNA was extracted from the cells and analyzed by Northern blot hybridization as described previously (20). Hybridizations were in $5 \times \text{SSPE}$, 0.1% SDS, 200 $\mu\text{g/ml}$ salmon sperm DNA, $5 \times$ Denhardt's solution at 65 °C with a murine *Spi-1* cDNA clone (13), which had been labeled with ^{32}P by random priming (21). Washes were to a final stringency of 62 °C in $0.1 \times \text{SSC}$, 0.1% SDS. Blots were developed by autoradiography and quantitated by densitometry utilizing a Bio-Rad model 620 video densitometer. RNA loading was standardized for the amount of 18 S rRNA loaded per lane, as determined by hybridization with a labeled fragment of the mouse 18 S rRNA gene (22) followed by autoradiography and densitometry, as above.

Expression of the recombinant *Spi-1* transcripts were determined by blot hybridization of RNAs separated by electrophoresis in 3.5% polyacrylamide gels, as described by Stoeckle and Guan (23), or by RNase protection. For the RNase protections, a 271-base pair *Bam*HI-*Xba*I fragment containing the bovine growth hormone 3'-UT sequences and polyadenylation signal from the pRC/RSV expression vector was used since RNA transcripts that included 3'-*Spi-1* sequences were poorly digested with either RNase A and T1 or RNase One (Promega), presumably due to stable secondary structure in this GC-rich (62%) sequence (13). The 3'-UT sequences were ligated between the *Bam*HI-*Xba*I sites in pBlueScript II SK and ^{32}P -labeled antisense RNAs transcribed with T7 RNA polymerase, as described by the manufacturer (Stratagene). 300,000–600,000 dpm of this radiolabeled RNA was hybridized with 15 μg of cytoplasmic RNA for 18 h at 60 °C in 0.4 M sodium chloride, 40 mM PIPES, 1 mM EDTA, 80% formamide. The unhybridized RNA was digested to completion with RNase One as described by the manufacturer (Promega), and the undigested fragments were separated by electrophoresis in denaturing 6% polyacrylamide gels and visualized by autoradiography.

Nuclear Run-off Transcriptions—Run-off transcriptions were performed on isolated nuclei as described previously (20). The run-off transcripts were isolated by centrifugation through 5.7 M cesium chloride, resuspended in diethyl pyrocarbonate-treated water, and standardized for cpm/ml by scintillation counting. Equal amounts of radioactivity were hybridized at 65 °C in TES-buffered 1 M sodium chloride and 1% SDS for 48 h with 10 μg of linearized plasmid or 5 μg of single-stranded phagemid DNA, which had been bound to nitrocellulose membranes by slot blotting. Following hybridization, the blots were washed at 65 °C in $2 \times \text{SSC}$, 0.1% SDS with a final wash in $2 \times \text{SSC}$ with RNase A added at 10 $\mu\text{g/ml}$. Results were visualized by autoradiography.

The DNAs used in these experiments included cloned DNAs encoding hsc70 (24), β -actin (25), β -globin (26), Band 3 (27), GATA-1 (28), NF-E2 (29), and *Spi-1* (13). The cloning vectors for these cDNAs, pUC19, pBlueScript II, and pGEMM were used as negative controls. For determination of sense and antisense transcription, pBlueScript II SK(+) and pBlueScript II SK(−) phagemids containing the respective cDNAs were rescued by infection with VCS-M13 interference-resistant helper phage. Virus was isolated from the supernatants by precipitation with polyethylene glycol as described by Stratagene. Phage DNA was purified by phenol and chloroform extraction and ethanol precipitation. For the experiments requiring probes specific to the 3'-end of the *Spi-1* transcript, a 250-base pair fragment of 3'-untranslated *Spi-1* sequence

was excised by digestion with *Apa*I and *Xho*I. The fragment was isolated by agarose gel electrophoresis and ligated into the polylinker of pBlueScript II SK(+) at the *Apa*I and *Xho*I sites.

Determination of *Spi-1* mRNA Half-life—The transcriptional inhibitor, DRB, was utilized at a concentration of 30 $\mu\text{g/ml}$ as described previously (30). This concentration has been shown to inhibit transcription by greater than 90% (31). MEL cells were grown with or without Me_2SO , and, following 24 h of incubation, DRB was added to the cells and incubations continued for an additional 4 h. Aliquots of cells were removed at hourly intervals following the addition of DRB, and RNA was extracted as described above. 10 μg of RNA from each sample was analyzed for expression of *Spi-1* by Northern blotting. Loading of RNA was standardized by hybridization with a radiolabeled 18 S rRNA probe. Autoradiographs were quantitated by densitometry with the results from each lane normalized to the amount of 18 S rRNA. *Spi-1* mRNA half-life was calculated by curve-fitting assuming linear decay, using the program included in the graphics software (Cricket Graph, Philadelphia, PA).

Expression of Recombinant DNA Constructs—The cDNA clone encoding *Spi-1* has been previously described (13). For expression in MEL cells, a *Spi-1* cDNA, which had truncations of both the 5'- and 3'-noncoding sequences, was prepared by polymerase chain reaction. Reactions were carried out in 50 mM potassium chloride, 1.5 mM magnesium chloride, 10 mM Tris, pH 7.6. The primers and linearized plasmid DNA were denatured at 94 °C for 1 min and annealed at 60 °C for 2 min, with extension at 72 °C for 3 min and denaturation at 94 °C for 1 min. This was repeated for 35 cycles in an MJ Research minicycler. The 5'-primer, GGATCTGACCAAACTAGTGCTCAGC, contained three point mutations at the underlined positions, creating a unique *Spe*I restriction site. The 3'-primer, GAGCCTGGCGGCCGCTGC, contained two point mutations at the underlined positions, creating a unique *Not*I site. When cut with *Spe*I and *Not*I, this cDNA contained 10 nucleotides of 5'- and 3'-untranslated sequences, proximal to the coding region. This 5'- and 3'-truncated cDNA was ligated into the *Spe*I and *Not*I sites in the pRC/RSV expression vector (Invitrogen), introduced into MEL cells by electroporation and stable transfectants selected in G418 (0.6 mg/ml, specific activity) as described previously (32). Based on previous determinations of stable cloning efficiency with pRC/RSV (approximately 1 per 5×10^4 electroporated cells), cells were seeded in 24-well dishes at concentrations that resulted in the outgrowth of resistant cell "pools," which represented the expansion of one to three original cell clones. The expanded pools were screened for expression of the recombinant mRNA by Northern blotting or RNase protection, and those expressing detectable levels were subjected to further analysis.

RESULTS

Post-transcriptional Regulation of *Spi-1* Expression in Inducer-exposed Erythroleukemia Cells—Previous experiments have demonstrated that Me_2SO exposure of MEL cells decreases expression of *Spi-1* (13, 15). This effect was examined for the MEL cell clones used in these experiments. The decrease in expression of *Spi-1* was detectable by 4 h of Me_2SO exposure and continued to decline until reaching 25–30% of control levels at 20–36 h of inducer exposure (Fig. 1). This decrease entirely preceded the increase in expression of β -globin mRNA, which was first apparent at 24–36 h. In contrast to the early decrease in *Spi-1* expression and the subsequent increase in expression of β -globin, the expression of two abundant "house-keeping" mRNAs, β -actin and ribosomal protein L26, was not significantly altered following inducer exposure. Thus, the effect of Me_2SO on *Spi-1* expression appeared to be selective.

To determine if the decreased abundance of *Spi-1* mRNA was due to a decrease in its transcription, run-off transcriptions were performed on isolated nuclei from cells grown under normal conditions or following 24 h of Me_2SO exposure. Since previous studies had demonstrated that antisense transcription occurs across some genes in MEL cells (including *c-myc* (33) and hsc70 (32)), the labeled run-off transcripts were hybridized to single-stranded sense and antisense cDNA probes for *Spi-1*. As demonstrated in Fig. 2A, a low level of antisense transcription (detected by hybridization with the cDNA encoding the positive strand) is detected across the *Spi-1* gene in these cells. This antisense transcription appears authentic,

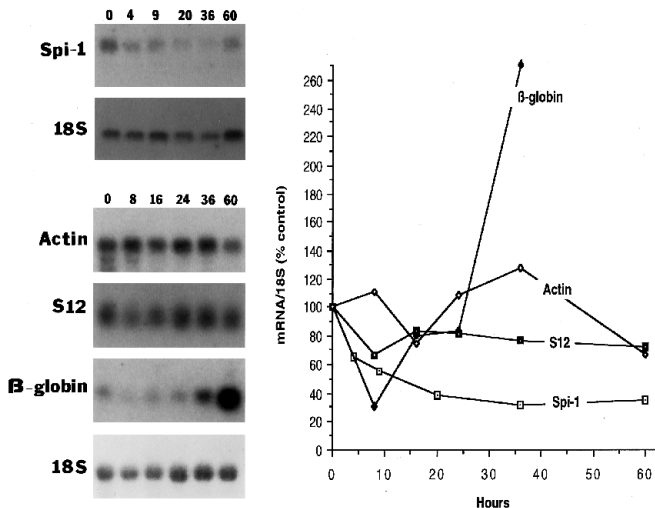


FIG. 1. **Me₂SO exposure causes a selective decrease in Spi-1 mRNA abundance.** MEL cells were grown in the presence of Me₂SO, and cytoplasmic mRNA was extracted at the indicated times. Equal amounts of RNA were separated by gel electrophoresis, and mRNA levels were determined by Northern blot hybridization with ³²P-labeled cDNAs encoding Spi-1, β -actin, ribosomal protein S12, and β -globin. Results were quantitated by densitometry and standardized for loading by normalizing to results obtained by rehybridizing the blots with a labeled fragment of the 18 S rRNA gene.

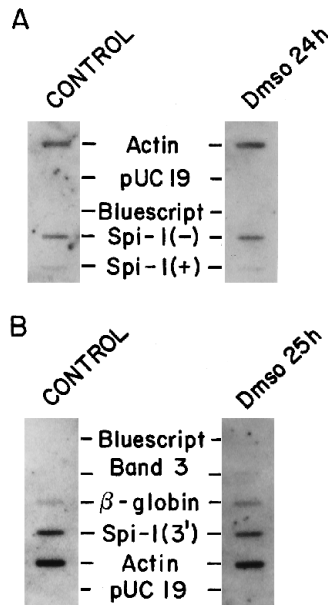


FIG. 2. **Me₂SO exposure does not affect transcription rate nor cause transcriptional attenuation of Spi-1.** A, run-off transcriptions were performed on nuclei prepared from control and Me₂SO-exposed cells as described in the text. Antisense Spi-1 transcripts were detected by hybridization of the labeled transcripts with single-stranded phagemid DNA containing the Spi-1 sense (+) strand. Sense transcripts were detected by hybridization with single-stranded phagemid containing the Spi-1 antisense (-) strand. The remaining DNAs used in this experiment were double-stranded. The plasmid DNAs pUC19 and pBlueScript II SK (+) were included as negative controls. B, run-off transcriptions were performed as above, and the labeled transcripts were hybridized with a cloned Spi-1 cDNA, which contained only the 3'-terminal 250 nucleotides of the mature transcript. Other plasmids included cDNAs with sequences encoding band 3, β -globin, and β -actin. The plasmid DNAs, pUC19 and pBlueScript II, were included as negative controls.

since no signal is detected hybridizing with the phagemid vector. The sense strand (hybridizing to the cDNA encoding the negative strand) is transcribed at approximately 10-fold the rate of the antisense transcript. However, as demonstrated

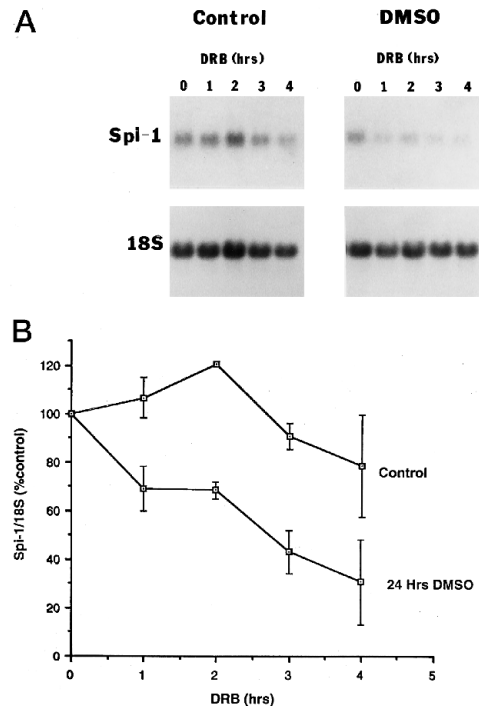


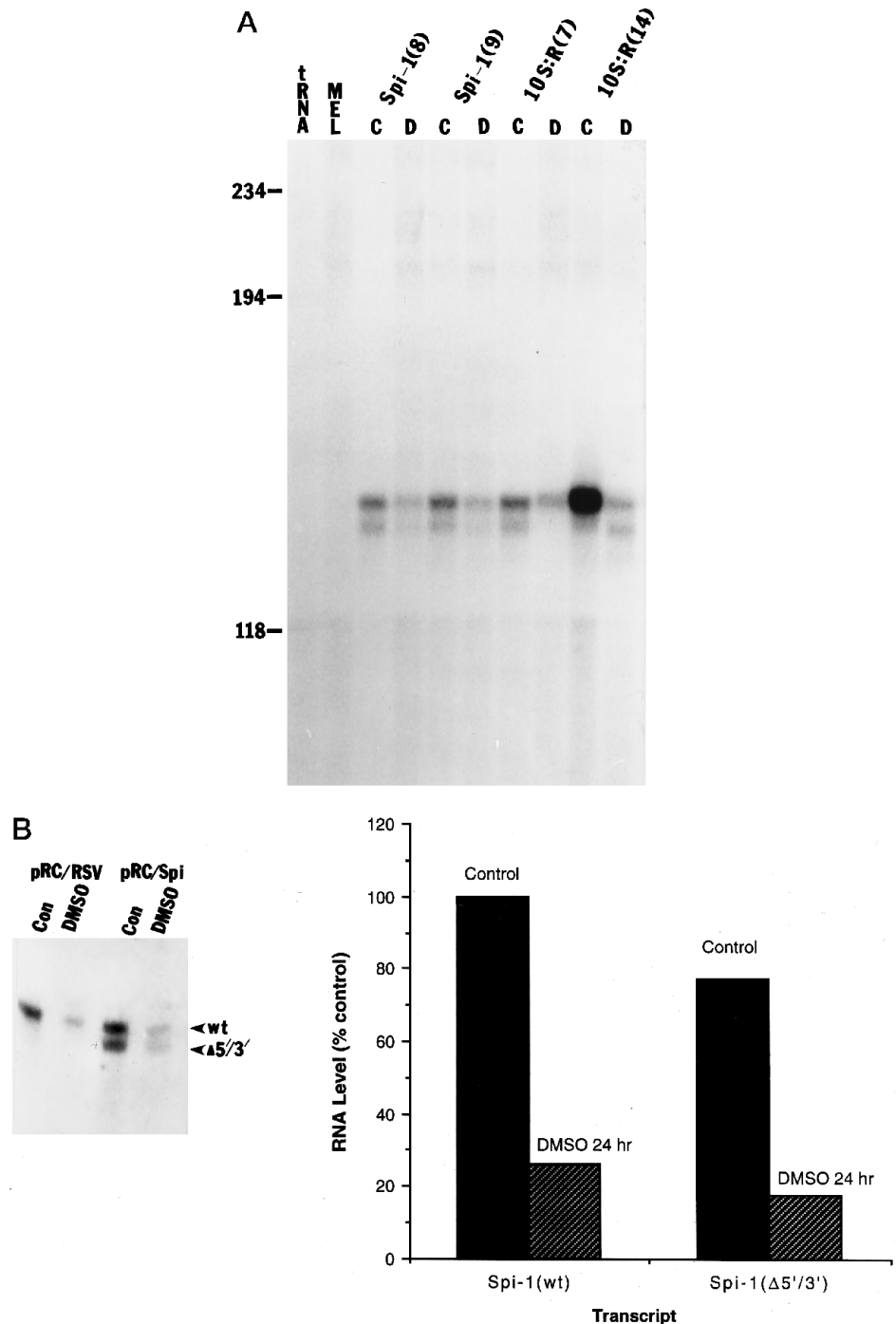
FIG. 3. **Me₂SO decreases Spi-1 mRNA half-life.** MEL cells were grown in the presence or absence of Me₂SO for 24 h; transcription was then inhibited by the addition of DRB, and the cells were incubated for an additional 4 h. RNA was extracted from the cells at 1-h intervals, and the Spi-1 mRNA level was determined by Northern blot hybridization. The experiments were performed in duplicate, and the results were quantitated by densitometry and standardized for loading by normalizing to results obtained by rehybridizing the blots with a cloned fragment of the 18 S rRNA gene. Representative blots are shown in panel A, and the results of the densitometric quantitation are shown in panel B. The results are expressed as a percentage of Spi-1 mRNA present at time 0, prior to the addition of DRB. The vertical bars in panel B represent the range for each determination.

here, neither sense nor antisense transcription rate is significantly altered following inducer exposure.

The early decrease in expression of *c-myc* mRNA that follows Me₂SO exposure of MEL cells is due to transcriptional attenuation (34). To determine if Me₂SO caused attenuation of Spi-1 transcription, the run-off transcriptions were repeated and hybridized with a cDNA fragment of Spi-1, which included only the 3'-UT sequences of this mRNA. Transcripts extending to the 3'-UT of this gene were equally represented in the run-off transcripts from both control and Me₂SO-exposed cells (Fig. 2B), demonstrating that attenuation of transcription had not occurred on this gene. These assays were sensitive to changes in transcription, since the transcription of β -globin at this time had increased from 3 to 17% of the rate actin transcription. The transcription of band 3 also appeared to increase, although this could not be quantified due to the lack of a detectable signal in the control cells. These results demonstrate that the transcription of Spi-1 in MEL cells was unaffected by Me₂SO exposure, and thus the decreased accumulation of this mRNA was mediated post-transcriptionally.

To determine the half-life of Spi-1 mRNA following Me₂SO exposure, cells were exposed to DRB, a specific inhibitor of RNA polymerase II transcription, since actinomycin D has previously been shown to induce MEL cell differentiation (35). We analyzed mRNA half-life following 24 h of inducer exposure, since the full extent of the decrease in expression of Spi-1 was evident by this time (see Fig. 1). Cells were grown in the presence or absence of Me₂SO for 24 h, then DRB was added for an additional 4 h. Cytoplasmic RNA was extracted at hourly

FIG. 4. Me₂SO exposure decreases the expression of a Spi-1 mRNA transcribed by the RSV promoter. A Spi-1 cDNA containing 10 nucleotides of 5'- and 3'-untranslated sequence under the transcriptional control of the RSV promoter was introduced into MEL cells, and stable clones expressing the recombinant transcript were selected in G418. A, the effect of Me₂SO on the expression of the recombinant transcript in four of these clones was determined by RNase protection, utilizing a probe that hybridized with the unique 3'-UT sequences derived from the vector. Each lane represents the results of a hybridization with 20 μ g of cytoplasmic RNA from either the parental cell line (MEL) or from the cloned cells (indicated by name above each paired set of lanes). Nuclease protections were performed with RNA from uninduced cells (C) and following 24 h of Me₂SO exposure (D). A protected fragment of the correct predicted size (135 nucleotides) was detected in the selected clones but not in the parental cell line. The smaller fragment results from the use of an alternative cleavage and polyadenylation site in the vector. B, the effect of Me₂SO on the expression of both recombinant and endogenous Spi-1 mRNAs was determined by electrophoresis of RNAs in 3.5% acrylamide gels and blot hybridization as described in the text. The results for clone pRC/Spi-1(8) are shown in the panel. Endogenous Spi-1 transcripts (*wt*) and the recombinant transcripts ($\Delta 5'/3'$) are indicated by the arrows to the right of the autoradiograph. Expression of the recombinant transcripts in two other analyzed clones exhibited a similar change.



intervals following the addition of DRB, and Spi-1 mRNA levels were determined by Northern blotting. The results shown in Fig. 3 are normalized for RNA loading as determined by rehybridizing the blots with an 18 S rRNA fragment and represent the average of two separate experiments. For control cells, the half-life of Spi-1 mRNA was determined to be 8.2 h, while following 24 h of Me₂SO exposure the half-life was reduced to 3 h. The decay was noted to be biphasic for both control and Me₂SO-treated cells, with the initial decrease (at 1–2 h of DRB exposure) accounting for the entire difference in half-life. Other investigators have also noted biphasic mRNA decay following exposure to actinomycin D (30), suggesting that a secondary response to transcriptional inhibitors accounted for this latter rate. While the measured decrease in half-life difference was slightly less than the 3–4-fold decrease in Spi-1 mRNA abun-

dance that followed inducer exposure, these results supported the conclusion that the stability of Spi-1 mRNA was decreased by inducer exposure.

Expression of a Recombinant Spi-1 cDNA Is Regulated Identically to Endogenous Spi-1—To provide independent confirmation of the preceding results, a Spi-1 cDNA, which included the entire coding sequence and 10 bases of 5'- and 3'-UT sequences, was ligated into an expression vector under the transcriptional control of the constitutively active RSV promoter. The terminal 130 nucleotides of 3'-UT and the polyadenylation signal were derived from the vector. This construct was introduced into MEL cells by electroporation, and stable cell lines were selected and analyzed for expression of the recombinant Spi-1 gene by RNase protection or Northern blotting of extracted RNAs. Clones with detectable expression of the recombinant construct

were analyzed further.

The effect of Me₂SO on expression of the recombinant transcript was determined on eight independent clones that expressed detectable levels of the recombinant transcript. The results of nuclease protection assays utilizing a probe derived from the 3'-UT sequences of the vector are shown for four of these clones (Fig. 4A). A fragment of 135 nucleotides, which corresponded to the expected length of the 3'-UT of the recombinant transcript, was detected in all the clones. The smaller fragment resulted from use of an alternative cleavage and polyadenylation site present in the 3'-UT (36). Hybridizing sequences were not detected in control cells. Exposure to Me₂SO (24 h) resulted in decreased expression of the recombinant transcript in all analyzed clones. To compare this decrease with that of endogenous Spi-1 mRNA, clone pRC/Spi-1(8) was analyzed by blot hybridization of RNA separated in 3.5% denaturing acrylamide gels. As demonstrated in Fig. 4B, following 24 h of Me₂SO exposure the extent of the decrease in expression of the recombinant mRNA was identical to that of the endogenous mRNA. Similar behavior was observed on two

other clones similarly screened (not shown).

The conclusion that expression of the recombinant Spi-1 transcript was regulated post-transcriptionally was inferred from evidence that the RSV promoter is constitutively active under these conditions, as determined by transient assays.² However, to confirm this, the effect of Me₂SO on transcription of the recombinant Spi-1 gene in clone pRC/Spi-1(8) was determined by run-off transcriptions. The vector (pRC/RSV) without inserted cDNA sequences was used to detect the 3'-sequences uniquely present in the recombinant transcripts. Run-off transcripts that hybridized to vector sequences were easily detected in the transfected clone, and their transcription was unaffected by Me₂SO (Fig. 5). Similar results were found with analysis of an additional clone. There was no significant hybridization detected with the pRC/RSV vector sequences in the parental cell line (see Fig. 6C). A weak signal was detected hybridizing to the plasmid DNA, pUC19, in the electroporated cells. This was detected in repeated experiments and was also noted for pGEMM plasmid DNA (data not shown). Since this was not detected in the nuclear run-off transcriptions performed on untransfected cells (see Figs. 1 and 2), this may represent hybridization with transcripts from the expression vector that extended into the plasmid backbone, since transcription past the cleavage and polyadenylation site would be an expected occurrence. These experiments confirmed that transcription from the vector was constant and unaffected by Me₂SO exposure. Therefore, the decreased expression of the recombinant Spi-1 mRNAs detected in the preceding experiments must be mediated post-transcriptionally.

Erythroid Transcription Factor mRNAs Are Not Destabilized by Me₂SO Exposure—To determine if Me₂SO had a similar effect on stability of other transcription factor mRNAs we analyzed the expression of two erythroid transcription factors, GATA-1 and NF-E2. As determined by Northern blot hybridization, Me₂SO had a minimal effect on the expression of GATA-1, although a transient decrease in mRNA level was noted at early times of inducer exposure (Fig. 6). NF-E2 also demonstrated a transient decrease in mRNA level followed by a modest induction, most evident at 72 h of inducer exposure

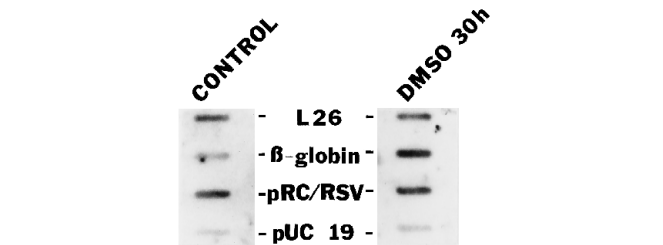


FIG. 5. **Transcription of the recombinant Spi-1 transcript is unaffected by Me₂SO exposure.** Transcriptional activity of the RSV promoter was determined for the clone shown in Fig. 4B by nuclear run-off transcription in cells grown with and without Me₂SO (1.5%) for 30 h as described in the text. Labeled transcripts were hybridized with 10 μ g of linearized plasmid DNAs. Plasmids containing cDNA inserts encoding β -globin and the ribosomal protein L26 or the plasmid pUC 19, without inserted sequences, were included as controls. Spi-1 transcripts derived from the expression vector were detected by hybridization with 3'-UT sequences present in the pRC/RSV vector to avoid detection of endogenous Spi-1 transcripts. Hybridization with pRC/RSV sequences was not detected in run-off transcriptions done on the parental cell line MEL cells (see Fig. 6C).

² D. L. Galson, unpublished data.

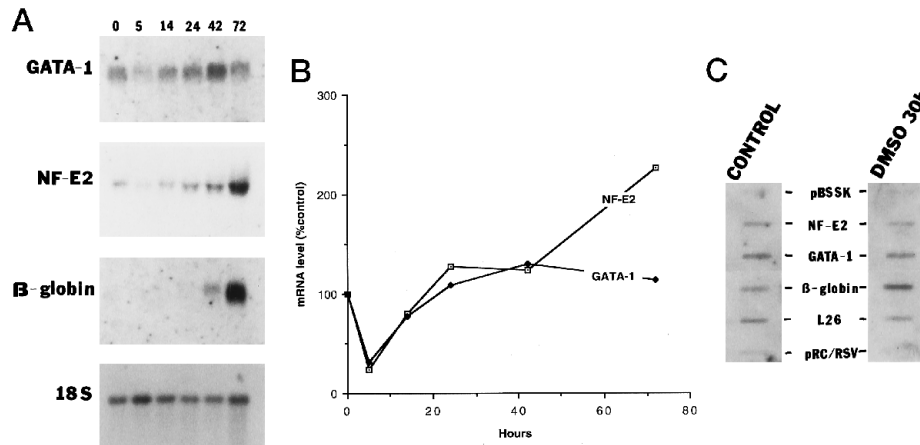


FIG. 6. **The stability of GATA-1 and NF-E2 mRNAs are unaffected by Me₂SO exposure.** A, RNA was extracted from MEL cells at the times of Me₂SO exposure indicated, and expression of GATA-1 and NF-E2 mRNA assessed by Northern blot hybridization is shown. β -globin mRNA expression was similarly assessed. B, the results were quantitated by densitometry and normalized for loading by hybridization with an 18 S rRNA gene fragment. The results are presented graphically at the right of the figure. The increase in β -globin gene expression was not quantified, since expression in uninduced cells was below the limits of detection by densitometry. C, nuclear run-off transcriptions were performed on uninduced cells and at 30 h of Me₂SO exposure. The preparation of nuclei and ³²P labeling of run-off transcripts were performed as described in the text. The labeled transcripts were hybridized with 10 μ g of slot-blotted plasmid DNAs with inserted cDNA sequences encoding r-protein L26 and β -globin or with single-stranded phagemids with inserted sequences corresponding to GATA-1 and NF-E2 antisense strands to detect hybridization with the sense transcripts of these genes. Phagemid DNA without inserted sequences (pBSSK) was included as control. The plasmid pRC/RSV was also included as a control for the run-off transcriptions performed on the electroporated cell lines shown in Fig. 5.

(Fig. 6B). A similar early, transient decrease in expression of other mRNAs has been noted in inducer-exposed cells, including mRNAs encoding *c-myc* (41) and *c-myb* (42) as well as β_{min} -globin (see Fig. 1).³ However, the failure to observe a persistent decrease in expression of GATA-1 and NF-E2 mRNAs suggested that their stability was unaffected by inducer exposure.

The transient decrease in GATA-1 and NF-E2 mRNA levels that followed Me₂SO exposure suggested that a decrease in the stability of these mRNAs could have been offset by a subsequent increase in their transcription. To determine if this was the case, we assessed the transcription of these genes in control cells and at 30 h of Me₂SO exposure by nuclear run-off transcription. At this time of inducer exposure, GATA-1 and NF-E2 mRNAs were expressed at approximately 110 and 125% of their respective levels in uninduced cells. Run-off transcriptions demonstrated that at 30 h of Me₂SO exposure, the transcription of these genes was unchanged relative to their transcription rates in the control cells. In contrast, an increase in transcription of β -globin was clearly evident at this time. These results demonstrate that the stability of these two mRNAs was not significantly altered by Me₂SO exposure. Therefore, the decrease in Spi-1 mRNA stability that occurs following inducer exposure is not the result of a global decrease in stability but exhibits specificity since neither GATA-1 nor NF-E2 mRNAs are similarly affected.

DISCUSSION

Exposure of MEL cells to the inducer of differentiation, Me₂SO, causes a 3–5-fold decrease in accumulation of mRNA encoding the transcription factor Spi-1 (13, 19). The data presented here demonstrate that post-transcriptional regulatory mechanisms are responsible for this decrease. In contrast, the stability of mRNAs encoding the transcription factors, GATA-1 and NF-E2, is unaffected by inducer exposure, demonstrating specificity to this regulation. These findings provide insight into the regulation of Spi-1 expression and suggest that differential stability of transcription factor mRNAs following inducer exposure may play a role in establishing the pattern of gene expression in the differentiating cells.

Exposure of MEL cells to Me₂SO results in a 70–75% decrease in accumulation of Spi-1 mRNA. Nuclear run-off transcriptions demonstrated that this decrease was mediated post-transcriptionally. Since increased expression of Spi-1 in Friend virus-transformed cells is due to retroviral insertional activation of transcription, these results suggest that the transcriptional activity of the retroviral long terminal repeat is unaffected by Me₂SO, and results of transient transfection assays have reached a similar conclusion.⁴ Thus, the decreased expression of Spi-1 is regulated post-transcriptionally. While the data presented here do not exclude that Me₂SO blocked nucleocytoplasmic transport of this mRNA, this appears unlikely since Schuetze *et al.* (19) detected a similar decrease in Spi-1 expression when whole cell mRNA was analyzed.

Determination of Spi-1 mRNA half-life by transcriptional inhibition demonstrated that Me₂SO exposure decreased the stability of Spi-1 mRNA. Since the expression of a recombinant Spi-1 cDNA transcribed by the constitutively active RSV promoter was regulated similarly to endogenous Spi-1, this limits the sequence required for this regulated change in stability to the coding region plus the adjoining 10 nucleotides of 5'- and 3'-untranslated sequences. The coding region of a number of other mRNAs, including β -tubulin, *c-fos*, and *c-myc*, has also

been shown to play a role in determining stability (43–46). The mechanisms by which these sequences regulate mRNA stability remain to be established. However, the ability of inducers of differentiation to regulate the effect of these elements provides an additional means to investigate these mechanisms.

The degradation of mRNAs is frequently preceded by their deadenylation (30, 37–40), and this is consistent with our recent observation that the inducers Me₂SO, hypoxanthine, and A23187 all cause substantial deadenylation of mRNAs.⁵ Spi-1 is one of the mRNAs so affected. In contrast, for two “house-keeping” mRNAs (specifically ribosomal protein L26 and S12), inducer exposure affected neither abundance nor poly(A) tail length. Although the mechanisms resulting in this selectivity of deadenylation are unknown, this likely plays a role in the differential stabilities of these mRNAs following inducer exposure. Current studies are assessing the effect of Me₂SO on the adenylation of GATA-1 and NF-E2 mRNAs.

Regardless of the mechanism(s) that account for the differential stability of Spi-1 and GATA-1 and NF-E2 mRNAs in inducer-exposed MEL cells, these differences are likely to be significant for the regulation of gene expression in differentiating cells. While the Me₂SO-induced decrease in expression of Spi-1 has not yet been demonstrated to play a role in MEL cell differentiation, the consistent overexpression of Spi-1 during leukemogenesis of these cells suggests that this decrease is unlikely to be without effect. Further, the ability of Me₂SO to destabilize mRNAs is unlikely to be limited to Spi-1. It is noteworthy that expression of a recombinant Id transcript in MEL cells has been reported to be decreased by Me₂SO (48), suggesting that Me₂SO also destabilizes this mRNA. The stability of GATA-1 and NF-E2 following inducer exposure has increased significance in light of evidence suggesting that the activity of GATA-1 containing promoters can be regulated by competition for binding with other transcription factors (49, 50). Thus, the ability of Me₂SO to destabilize Spi-1 or other, yet to be identified transcription factor mRNAs may lead to a functional increase in activity of those mRNAs unaffected by this change. Further knowledge of how inducers affect mRNA stability should provide additional insights into the mechanisms by which these agents cause differentiation of these cells.

Acknowledgments—We thank Fritz Rottman for helpful discussions and the following individuals for the cloned DNA fragments used for these experiments: L. Giehl, B. Spiegelman, S. Alper, S. Orkin, and I. Wool.

REFERENCES

- Ben-David, Y., and Bernstein, A. (1991) *Cell* **66**, 831–834
- Li, J.-P., D'Andrea, A., Lodish, H., and Baltimore, D. (1990) *Nature* **343**, 762–764
- Lavigne, A., Cheong, G., and Bernstein, A. (1990) *New Biol.* **2**, 1015–1023
- Peacock, J., and Benichou, S. (1990) *Mol. Cell. Biol.* **10**, 3307–3313
- Moreau-Gachelin, F., Tavittian, A., and Tambourin, P. (1988) *Nature* **331**, 277–280
- Moreau-Gachelin, F., Ray, D., Mattei, M.-G., Tambourin, P., and Tavittian, A. (1989) *Oncogene* **4**, 1449–1456
- Goebel, M., Moreau-Gachelin, F., Ray, D., Tambourin, P., Tavittian, A., Klemsz, M., McKercher, S., Celada, A., van Beveren, C., and Maki, R. (1990) *Cell* **61**, 1165–1166
- Galson, D., and Housman, D. (1988) *Mol. Cell. Biol.* **8**, 381–392
- Klemsz, M., McKercher, S., Celada, A., van Beveren, C., and Maki, R. (1990) *Cell* **61**, 113–124
- Kominata, Y., Galson, D., Waterman, W., Webb, A., and Auron, P. (1995) *Mol. Cell. Biol.* **15**, 59–68
- Pongubala, J., Nagulapalli, S., Klemsz, M., McKercher, S., Maki, R., and Atchison, M. (1992) *Mol. Cell. Biol.* **12**, 368–378
- Zheng, D.-E., Hetherington, C., Chen, H.-M., and Tenen, D. (1994) *Mol. Cell. Biol.* **14**, 373–381
- Galson, D., Hensold, J., Bishop, T., Schalling, M., D'Andrea, A., Jones, C., Auron, P., and Housman, D. (1993) *Mol. Cell. Biol.* **13**, 2929–2941
- Scott, E., Simon, M., Anastasi, J., and Singh, H. (1994) *Science* **265**, 1573–1577

³ J. O. Hensold, C. A. Stratton, D. Barth, and D. L. Galson, unpublished observations.

⁴ D. L. Galson, unpublished observations.

⁵ J. O. Hensold, D. Barth, and C. A. Stratton, submitted for publication.

15. Schuetze, S., Steinberg, P., and Kabat, D. (1993) *Mol. Cell. Biol.* **13**, 5670–5678
16. Ben-David, Y. E. G., Letwin, K., and Bernstein, A. (1991) *Genes & Dev.* **5**, 908–918
17. Nunn, M., Seeburg, P., Moscivici, C., and Duesberg, P. (1983) *Nature* **306**, 391–395
18. Marks, P., and Rifkind, R. (1978) *Annu. Rev. Biochem.* **47**, 419–448
19. Schuetze, S., Paul, R., Gliniak, B., and Kabat, D. (1992) *Mol. Cell. Biol.* **12**, 2967–2975
20. Hensold, J., Dubyak, G., and Housman, D. (1991) *Blood* **77**, 1362–1370
21. Feinberg, A., and Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13
22. Wilson, G., Hollar, B., Waterson, J., and Schmekel, R. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 5367–5371
23. Stoeckle, M., and Guan, L. (1993) *BioTechniques* **15**, 227–229
24. Giebel, L., Dworniczak, B., and Bautz, E. (1988) *Dev. Biol.* **125**, 200–207
25. Spiegelman, B., Frank, M., and Green, H. (1983) *J. Biol. Chem.* **258**, 10083–10089
26. Tilghman, S., Tiemeier, D., Polsky, F., Edgell, M., Seidman, J., Leder, A., Enquist, L., Norman, B., and Leder, P. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 4406–4410
27. Kopito, R., and Lodish, H. (1985) *Nature* **316**, 234–238
28. Tsai, S.-F., Martin, D., Zon, L., D'Andrea, A., Wong, G., and Orkin, S. (1989) *Nature* **339**, 446–451
29. Andrews, N., Erdjument-Bromage, H., Davidson, M., Tempst, P., and Orkin, S. (1993) *Nature* **362**, 722–728
30. Laird-Offringa, I., de Wit, C., Elfferich, P., and van der Eb, A. (1990) *Mol. Cell. Biol.* **10**, 6132–6140
31. Laub, O., Jakobovits, E., and Aloni, Y. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 3297–3301
32. Hensold, J., Hunt, C., Calderwood, S., Housman, D., and Kingston, R. (1990) *Mol. Cell. Biol.* **10**, 1600–1608
33. Kindy, M., McCormack, J., Buckler, A., Levine, R., and Sonenshein, G. (1987) *Mol. Cell. Biol.* **7**, 2857–2862
34. Nepveu, A., Marcu, K., Skoultchi, A., and Lachman, H. (1987) *Genes & Dev.* **1**, 938–945
35. Ebert, P., Wars, I., and Buell, D. (1976) *Cancer Res.* **36**, 1809–1813
36. Woychik, R., Lyons, R., Post, L., and Rottman, F. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 3944–3948
37. Bernstein, P., and Ross, J. (1989) *Trends Biochem. Sci.* **14**, 373–377
38. Decker, C., and Parker, R. (1993) *Genes & Dev.* **7**, 1632–1643
39. Sachs, A. (1993) *Cell* **74**, 413–421
40. Shyu, A.-B., Belasco, J., and Greenberg, J. (1991) *Genes & Dev.* **5**, 221–231
41. Lachman, H., and Skoultchi, A. (1984) *Nature* **310**, 592–594
42. Ramsey, R., Ikeda, K., Rifkind, R., and Marks, P. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 6849–6853
43. Kabnick, K., and Housman, D. (1988) *Mol. Cell. Biol.* **8**, 3244–3250
44. Shyu, A., Greenberg, M., and Belasco, J. (1989) *Genes & Dev.* **3**, 60–72
45. Wisdom, R., and Lee, W. (1991) *Genes & Dev.* **5**, 232–243
46. Yen, T., Gay, D., Pachter, J., and Cleveland, D. (1988) *Mol. Cell. Biol.* **8**, 1224–1235
47. Deleted in proof
48. Shoji, W., Yamamoto, T., and Obinata, M. (1994) *J. Biol. Chem.* **269**, 5078–5084
49. Fischer, K., Haese, A., and Nowock, J. (1993) *J. Biol. Chem.* **268**, 23915–23923
50. Aird, W., Parvin, J., Sharp, P., and Rosenberg, R. (1994) *J. Biol. Chem.* **269**, 883–889