Spi-1/PU.1 Oncoprotein Affects Splicing Decisions in a Promoter Binding-dependent Manner*

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The expression of the Spi-1/PU.1 transcription factor is tightly regulated as a function of the hematopoietic lineage. It is required for myeloid and B lymphoid differentiation. When overexpressed in mice, Spi-1 is associated with the emergence of transformed proerythroblasts unable to differentiate. In the course of a project undertaken to characterize the oncogenic function of Spi-1, we found that Spi-1 interacts with proteins of the spliceosome in Spi-1-transformed proerythroblasts and participates in alternative splice site selection. Because Spi-1 is a transcription factor, it could be hypothesized that these two functions are coordinated. Here, we have developed a system allowing the characterization of transcription and splicing from a single target. It is shown that Spi-1 is able to regulate alternative splicing of a pre-mRNA for a gene whose transcription it regulates. Using a combination of Spi-1 mutants and Spi-1-dependent promoters, we demonstrate that Spi-1 must bind and transactivate a given promoter to favor the use of the proximal 5′ alternative site. This establishes that Spi-1 affects splicing decisions in a promoter binding-dependent manner. These results provide new insight into how Spi-1 may act in the blockage of differentiation by demonstrating that it can deregulate gene expression and also modify the nature of the products generated from target genes.

The transcription factor Spi-1/PU.1 plays an important role in the coordination of hematopoiesis. It is required for the development of both myeloid and B lymphoid lineages (1, 2). The level of its expression is accurately regulated as a function of hematopoietic development (3, 4). The relevance of the differential and tight regulation of spi-1 was demonstrated by the consequences of its deregulation in adult mice. Indeed, reduced Spi-1 expression can be associated with the development of acute myeloid leukemia (5–7). In contrast, spi-1 transcriptional activation exhibits an oncogenic activity in the erythroid lineage in which it is not normally expressed (8). To better understand the role of spi-1 overexpression in the development of erythroleukemia, spi-1 transgenic mice were generated (9). In these animals, forced Spi-1 activity blocks the differentiation of proerythroblasts, leading to the development of severe anemia and hepatosplenomegaly. Subsequently, proerythroblasts acquire an abnormal proliferation ability; resulting in the development of an erythroleukemia. Spi-1 belongs to the ETS family of transcription factors. Its ETS domain recognizes DNA on a 5′-(A/G)GAA-3′ core (10). In addition, Spi-1 contains an amino-terminal transactivation domain and a central PEST region (11). Spi-1 controls primarily the transcription of myeloid and lymphoid genes. Additionally, fli-1 is a direct target gene of Spi-1 in the erythroid tissue (12). The transcriptional activity of Spi-1 depends on its combinatorial association within multiprotein complexes. Some of these proteins are ubiquitous factors such as the basal transcription factor TFIIID (13) and the co-activator/integrator CREB-binding protein (cAMP-response element-binding protein) (14). Other Spi-1-interacting partners are tissue-specific, such as the B lymphoid factor NF-EM5/Pip (15, 16), the myeloid regulators c-JUN (17), AMLI and C/EBPα (18), MaB (19), and the erythroid transcription factor GATA-1 (20, 21).

Spi-1 has also been identified as a partner of proteins of the spliceosome. It participates in the choice of alternative splice sites by favoring the selection of the proximal 5′-splice site of E1A pre-mRNA (22). Translocated in liposarcoma (TLS)3 is one of the Spi-1 partners that is able to recognize RNA and to act in RNA splicing (22, 23). Spi-1 counterbalances the effect of TLS in the selection of alternative 5′-splice sites in erythroid cells. Its function in splicing as well as its interference with TLS requires the DNA binding domain (DBD) associated with the transactivation domain or the PEST region (24). The function of some splicing factors requires an ability to identify intronic or exonic RNA elements within pre-mRNAs. Although the DBD of Spi-1 is able to interact with poly(A) RNAs and homoribonucleotide poly(G) polymers (25), Spi-1 does not exhibit RNA recognition specificity (from a SELEX strategy).4 Thus, it is unlikely that the role of Spi-1 in splicing proceeds via recognition of a specific RNA sequence.

Transcription and splicing are coordinated events (26). This coordination appears to be mediated, in part, by the COOH-terminal domain of RNA polymerase II, which recruits splicing factors to transcription sites. Furthermore, it has been demonstrated that splicing of a transcript can be modified by changes in its promoter and transcribed sequence (27). These results are consistent with earlier observations that Spi-1 can cooperate with TLS to regulate splicing by interacting with TLS at a promoter fragment (28).

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3 The abbreviations used are: TLS, translocated in liposarcoma; DBD, DNA binding domain; CMV, cytomegalovirus; MT, Myc tag; RT, reverse transcriptase; EMSA, electrophoretic mobility shift assay; IRES, internal ribosome entry site; SR protein; Ser/Arg-rich protein; wt, wild-type.

4 A. Lerga and F. Moreau-Gachelin, unpublished data.
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in the promoter-driven transcription (27, 28). This led to the idea that transcription factors could regulate subsequent processing events. In fact, this notion has been further supported by the observation that several transcription factors interact with proteins of the spliceosome and/or display dual functions in splicing and transcription. Among these factors are p54

\text{splicing and transcription through the promoter has been described (32). Nuclear hormone receptors have also been shown to affect splicing decisions in a promoter-dependent manner (33). Indeed, alternatively spliced variants are selected according to the nature of co-activators recruited to the promoters by nuclear hormone receptors (34, 35). Because Spi-1 is also a transcription factor, it can be hypothesized that if the two Spi-1 functions are coordinated, the action of Spi-1 in splicing may proceed via DNA recognition. To examine this possibility, we investigated whether Spi-1 modifies the splicing of a pre-mRNA for a gene whose transcription it controls. Here, we provide evidence that the Spi-1 protein is able to modify alternative splicing of the E1A pre-mRNA expressed from Spi-1-dependent promoters. Using a combination of Spi-1 mutants and Spi-1-dependent promoters, it is shown that Spi-1 modifies splicing as a function of its ability to bind DNA. Indeed, Spi-1 must bind and transactivate a given promoter to favor the use of the proximal 5’ alternative site. Moreover, it is demonstrated that this effect is not due to the modulation of target mRNA transcription levels but depends on qualitative effect of Spi-1 in splicing. These results establish that Spi-1 affects splicing decisions in a promoter binding-dependent manner.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, penicillin/streptomycin, and 1-glutamine (Invitrogen).

Plasmid Constructs—pCS3-MT, pCS3-MT-Spi-1, and pCS3-E1A were previously described (22, 23). The CMV promoter contained in the pCS3 vector corresponds to the CMVIE94 sequence. pCS3-MT-DBD, pCS3-MT-Δ101, and pCS3-MT-ΔCter were generated by PCR amplification of the appropriate regions from the spi-1 cDNA and insertion in the pCS3-MT vector. The pCS3-MT-Δβ4 mutant, with amino acids 250–254 deleted in the pCS3-MT expression vector, was obtained by mutagenesis of the wild-type spi-1 cDNA using the QuickChange site-directed mutagenesis system (Stratagene) according to the manufacturer’s recommendations. The following mutagenic oligonucleotides were used for PCR: forward primer, 5’-GAAGAAAGTCAGAGAGAGACCGCGAGG-3’; reverse primer, 5’-CAGCACACTTGGCCGCTCTTCTTCTTCTATGTTTAC-3’. In all constructs, a nuclear localization signal and 6 copies of a Myc epitope (MT) were added to the NH2-terminal part of the proteins as mentioned in Ref. 22. The fes binding sites and the fli-1 (270/41) promoters were previously described (12, 36). In the case of the fes promoter, a thymidine kinase minimal promoter was added downstream from the fes target element. These sequences replaced the CMV promoter in the pCS3-E1A vector. The neomycin resistance sequence from pRESNeo (Clontech) was replaced by the firefly luciferase sequence derived from the pGL2 basic luciferase reporter (Promega). All clones were verified by sequencing. Detailed cloning procedures are available on request.

Transfections—Cells (0.5 × 10⁶) were transfected with Lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions. The Lipofectamine Plus/DNA mixture was left on cells for 5 h. The plasmid DNA quantities used were as follows: 100 ng for the pE1A-RES-LucF, 10 ng for the CMVE1A vectors, and 20–500 ng for the different Spi-1 expression vectors. When transcriptional or splicing effects of different amounts of reporter vectors or spi-1 expression vectors were compared, the total quantities of DNA were equalized using pBluescript (Stratagene) or pCS3-MT plasmids, respectively. Transfection efficiencies were normalized by co-transfection of a pCMV-Renilla luciferase (Promega) reporter vector (10 ng). The CMV promoter of the pCMV-Renilla luciferase vector does not contain any characterized or putative Spi-1 binding sites. Cells were harvested 24 h post-transfection. Transfected cells were separated into three parts: 1/5 to measure luciferase activity, 1/10 to analyze protein expression by Western blotting, and the rest was used to extract total RNA.

Luciferase Activity Measurement—Luciferase activity reflects the accumulation of RNA following both the transcription and degradation of RNA. Because the various spliced forms of E1A mRNA appear not to differ in terms of degradation (Ref. 37 and the comparison of RNA accumulation using monocistronic and bicistronic vectors), “transcriptional activity” in the text stands for RNA accumulation. Twenty-four hours post-transfection, 1/5 of the cell pellets were lysed and the firefly (LucF) and Renilla (LucR) luciferase activities were measured with the dual luciferase kit (Promega) according to the manufacturer’s instructions. The -fold induction of LucF was calculated after normalization to LucR activities.

RNA Purification and RT-PCR Analysis—Total RNA was prepared and treated with DNase I (Qiagen) as previously described (22). RNA was reverse transcribed with Moloney murine leukemia virus Superscript II reverse transcriptase (Invitrogen) in the presence of 50 μM dNTP and 2 pmol of 3’T E1A primer. PCR to study the splicing profile was performed with Taq DNA polymerase (PerkinElmer Life Sciences) in the presence of a 5’ E1A primer that was 5’ end-labeled with T4 polynucleotide kinase (Roche) and [γ-32P]ATP (Amersham Biosciences), as described elsewhere (22). The number of PCR cycles was kept to a minimum (18–22 cycles) to detect signals within the linear range of the assay. Control RT-PCR contained a RNA template that had not undergone reverse transcription. E1A RT-PCR products were resolved on 6% polyacrylamide-urea gels, autoradiographed, and quantified with ImageQuant on a GE Healthcare PhosphorImager. All transfection experiments were repeated at least three times. Semi-quantitative RT-PCR was performed with two independent dilutions of RNA that had been reverse-transcribed using primers amplifying all E1A forms (Fig. 1A, black arrows). The amount of reverse-transcribed RNA used for PCR was calculated as a
function of transfection efficiencies measured using LucR activity. The two RNA dilutions and the number of PCR cycles (30) used were within a proportional range of the assay. The sequences of the primers were: forward primer, 5'-TCAGCTGGTCCAAAAGACTG-3' and reverse primer, 5'-CAAGCTTGATTAGGTGGA-3'. RT-PCR products were resolved on 1% agarose gels and stained with ethidium bromide.

**Immunoblotting**—Proteins were boiled in sample loading buffer (62 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromphenol blue, 100 mM dithiothreitol), then separated by 10% SDS-PAGE and electrotransferred onto Hybond nitrocellulose membranes (Amersham Biosciences). Membranes were blocked with 5% nonfat dry milk in PBS, 0.1% Tween 20 (PBST) before incubation with the monoclonal 9E10 antibody directed against the Myc epitope (Santa Cruz Biochemicals, Santa Cruz, CA) in 5% nonfat dry milk/PBST. After three washes in PBST, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody and finally washed in PBS/Tween. The proteins were visualized with the enhanced chemiluminescence Western blotting detection system (Amersham Biosciences).

**Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)**—For the DNA binding assay, the various Spi-1 proteins were translated in *vitro* from CS3 vectors using TNT-coupled reticulocyte lysates (Promega). Nuclear extracts were prepared from 6 × 10⁶ transfected HeLa cells. Nuclei were collected by centrifugation after incubation for 20 min on ice in 200 μl of buffer containing 20 mM HEPES (pH 7.6), 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA (pH 7.5), 0.1% Triton X-100, 1% dithiothreitol, and protease inhibitor mixture tablets (Roche). The nuclei were resuspended in 80 μl of the same buffer containing 500 μl NaCl and incubated for 1 h on ice. The samples were centrifuged at 45,000 × g for 15 min at 4 °C to recover the supernatants corresponding to nuclear extracts.

The sequences of the wt *fes* (fes-wt) and mutated *fes* (fes-mut) probes were the following: *fes*-wt, 5'-GAGGAGGGCCGGGAA-TACAGGAACCTGCCCAGGCC-3', and *fes*-mut, 5'-GAGGAGGAGCAGGCCCACACAACTGGGCCCCGAGG-3'. The sequences of the CMV probes were: seqA, 5'-TATAGTATCTTCATATATGGTTTTCTATTGACG; seqB, 5'-CCATATGGGCTTCTTATACCGCCA; and seqC, 5'-TATATAGGTCTTTCTATTGACGTCAT. The nucleotides recognized by Spi-1 are in bold characters. The probes were 5'-labeled with T4 polynucleotide kinase (Roche) and [γ-³²P]ATP (5000 Ci/mmol). EMSA were performed as previously described (36). For the supershift assay, the monoclonal 9E10 antibody (Santa Cruz Biochemicals) was added to the binding reaction before addition of the probes. The DNA-protein complexes were submitted to electrophoresis on native 6% polyacrylamide gels in 0.5× Tris borate-EDTA (TBE) buffer and autoradiographed.

**RESULTS**

**Spi-1 Is Able to Control Splicing of a Pre-mRNA for a Gene Whose Transcription It Regulates**—To determine whether Spi-1 controls the splicing of a pre-mRNA for a gene whose transcription it controls, we developed a model system using a vector that expresses a bicistronic pre-mRNA encoding the E1A minigene and a luciferase gene. This vector (p*pes*-E1A-IRES-LucF or p*fli*-I-E1A-IRES-LucF) allows the characterization of transcription and RNA maturation simultaneously for the same gene. Two types of *spi-1* target promoters were used to control the mRNA expression; one derived from the myeloid *fes* gene (36) and the other from the hematopoietic *fli-1* gene (12). The *fes* box consists of 3 Spi-1 DNA binding sites plus the minimal thymidine kinase promoter (Fig. 1A). The *fli-1* sequence includes the minimal promoter of the *fli-1* gene containing 2 Spi-1 DNA binding sites (Fig. 2A). The *fes* or *fli-1* E1A-IRES-LucF construct was transfected together with increasing amounts of Spi-1 expression vector in HeLa cells, which do not express the genomic *spi-1* gene (data not shown and Fig. 3). For each sample, the luciferase activity was measured, the Spi-1 expression was analyzed by Western blot, and the E1A isoforms were amplified by radioactive RT-PCR, separated by electrophoresis, and quantified by phosphorimaging.

First, we controlled that the luciferase activity, encoded by the same mRNA molecule as the E1A, reflects the level of RNA transcribed. E1A transcription was analyzed by semi-quantitative RT-PCR of all E1A RNA isoforms, spliced or not (see the position of the primers in Fig. 1A), and compared with the luciferase activity. In all experiments, the luciferase activity and the quantity of RNA used for semi-quantitative RT-PCR were normalized to the transcription efficiency. The results obtained with the *fes* reporter vector are shown in Fig. 1B. An increase in E1A pre-mRNA transcription by Spi-1 as revealed by semi-quantitative RT-PCR (compare the band intensities for control cells to Spi-1-transfected cells) was correlated with an increase in luciferase activity (9-fold), establishing that the luciferase activity correctly reflects the amount of RNA transcribed. Thus, we used this system to further investigate Spi-1 function in transcription and splicing. As shown in Fig. 1B, Spi-1 enhanced transcription of the minigene driven by the *fes* box in a dose-dependent manner as measured by luciferase activities. The transcription was increased 9-, 13-, or 15-fold according to the amount of Spi-1 expression vector transfected. The presence of three alternative 5'-splice sites in the first exon of the E1A pre-mRNA gives rise to three major mRNA isoforms, 13 S, 12 S, and 9 S, transcribed from the E1A minigene (Fig. 1A). Two RNA isoforms (11 S and 10 S) were also generated that were not taken into account because they arise from double splicing events (38). Fig. 1C presents the splicing profiles of the E1A mRNA. The histograms represent the relative proportion of 13 S, 12 S, and 9 S E1A isoforms. The decrease in the proportion of the 9 S isoform in Spi-1-transfected HeLa cells relative to that observed in HeLa cells transfected with the empty vector were indicated under each histogram as % S decrease versus control. The increase of E1A minigene transcription by Spi-1 was associated with a decrease in the proportion of the E1A 9 S isoform (up to 70% compared with control cells), whereas the proportion of the 13 S and 12 S isoforms increased (Fig. 1C). These results revealed that Spi-1 promotes the use of the proximal 5'-splice site of an E1A pre-mRNA whose transcription is driven by a Spi-1-responsive promoter. Similar experiments were performed with the vector containing the *fli-1* promoter (Fig. 2). Once again, E1A transcription was enhanced in Spi-1-transfected HeLa cells up to 4.7-fold compared with cells trans-
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**FIGURE 1.** Transcriptional and splicing activities of the wild-type Spi-1 protein on the E1A minigene whose transcription is driven by Spi-1 responsive elements of the fes gene promoter. HeLa cells were co-transfected with increasing amounts of Spi-1 expression vector as indicated, 10 ng of the pCMV-LucR normalization vector, and 10 ng of the vector carrying the bicistronic pre-mRNA (p fes /fli-1 minigene under control of the fes or fli-1 promoters (data not shown). The variations in both luciferase activity and E1A splicing relative to Spi-1 expression levels were similar to those observed in the assay using the bicistronic vectors, demonstrating that the IRES did not interfere with the role of Spi-1 in transcription and splicing. Our results demonstrate that Spi-1 is able to regulate the alternative splicing of a gene whose transcription it regulates.

Splicing Effect of Spi-1 Is Determined by Its Ability to Bind to the Promoter Driving the Transcription of the Pre-mRNA—The fact that Spi-1 modulated the splicing of a pre-mRNA for a gene whose transcription it controls raised the possibility that Spi-1 binding to DNA was necessary to affect splicing decisions. Thus, to examine this question, we set up a strategy based on the swapping of promoters driving the transcription of the bicistronic pre-mRNA encoding the E1A minigene and the luciferase gene. Because we did not find mutations that completely abolished the binding of Spi-1 to the fli-1 promoter, we decided to focus on the fes promoter.

We have previously described the minimal fes recognition sequence of Spi-1 as 5′-AGGAA-3′, and shown that the replacement of the two Gly by two Cys abolished binding of Spi-1 (36). We performed EMSA using the fes-wt and fes-mut probes and nuclear extracts from transiently transfected HeLa cells with the Spi-1 expression vector (Fig. 3). As a control, Spi-1 protein generated in reticulocyte lysates was used. As seen in Fig. 3, Spi-1 that was contained in nuclear extract from HeLa cells transfected with an Spi-1 expression vector induced a shift of the fes-wt probe but not the fes-mut probe in agreement with the fact that Spi-1 did not bind to the mutated fes sequence, 5′-ACCAA-3′. Thus, a bicistronic expression vector containing three mutated fes oligonucleotides in the promoter was constructed (p fes-mut-E1A-IRES-LucF). It was used in transactivation and splicing assays to determine whether the effect of Spi-1 on splicing was different when it could no longer bind to the promoter.

The transcriptional activity of Spi-1 was measured in HeLa cells transfected with a Spi-1 expression vector and either the p fes-wt-E1A-IRES-LucF or p fes-mut-E1A-IRES-LucF target vector. As reported above, transcription levels were evaluated by semi-quantitative RT-PCR of E1A RNA and by luciferase activity. Spi-1 expression was determined in transfected cells by semi-quantitative RT-PCR of E1A RNA and by luciferase activity. Spi-1 expression was determined in transfected cells by Western blotting. It can be seen in Fig. 4A that the luciferase

unspliced E1A pre-mRNA (us), 13 S, 12 S, 11 S, 10 S, and 9 S RNAs are indicated. Autoradiograms of the radiolabeled PCR products obtained in a representative experiment are shown. Histograms represent the percentage of unspliced E1A RNA in transfected cells relative to the percentage of the 9 S isoform in cells transfected with the empty vector. Mean ± S.D. were calculated from three independent experiments with duplicate samples.
activity was enhanced 7-fold by Spi-1 when the transcription was driven by the \textit{fes}-wt promoter, whereas it was similar in the absence and presence of Spi-1 when transcription of luciferase was driven by the \textit{fes}-mut promoter (Fig. 4A). Similarly, semi-quantitative RT-PCR of E1A RNA showed that Spi-1 increased E1A RNA expression from p\textit{fes}-wt-E1A-IRES but not from the p\textit{fes}-mut-E1A-IRESLucF vector (Fig. 4A). Similar results were obtained with the monocistronic vectors (data not shown).

Next, we investigated the splicing effect of Spi-1 on E1A in the same transfected samples. All the experiments presented used the bicistronic E1A vector. As described in Fig. 1, when the E1A minigene was transcribed from the \textit{fes}-wt promoter, Spi-1 modified the splicing pattern, resulting in a decrease in the proportion of the 9 S isoform (up to 40% of the 9 S proportion found in control cells), and an increase in the 13 S RNA isoforms (Fig. 4B, left part). When transcription of the E1A minigene was driven by the \textit{fes}-mut promoter, Spi-1 did not favor the use of the most proximal 5' alternative site but slightly reinforced the use of the distal 5' splice site (Fig. 4B, right part), as deduced from the 20% increase in the proportion of the 9 S isoform compared with the control. These results demonstrate that Spi-1 affects splicing decisions as a function of its ability to bind to the promoter.

It should be noted that the effects of Spi-1 on the splicing of the E1A target whose transcription was driven by the \textit{fes} and \textit{fli-1} promoters was qualitatively similar to those previously
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observed for the E1A target whose transcription was driven by CMV (24). In view of the different Spi-1 splicing effects described here, it was necessary to further characterize the Spi-1 splicing effect on CMVE1A pre-mRNA. First, we analyzed whether Spi-1 was able to bind to the CMV promoter by EMSA. Eight putative Spi-1 binding sites, 5′-(A/G)GAA-3′, were identified in the CMV promoter. EMSA was performed using 3 different probes containing a total of 4 putative Spi-1 binding sites. As seen in Fig. 5A, Spi-1 induced a shift of two of the three probes tested. HeLa cells were co-transfected with 10 ng of the CMVE1A vector and 500 ng of the Spi-1 expression vector or an empty vector. Then, the accumulation of RNA in the presence of Spi-1 was measured by semi-quantitative RT-PCR of E1A pre-mRNA as described above (Fig. 5B). A 2-fold increase in E1A RNA accumulation was observed. As previously described (24), Spi-1 modified the splicing pattern resulting in a decrease in the proportion of the 9 S isoform (Fig. 5C, 30% of the 9 S proportion found in the control cells). Consequently, these results are consistent with our demonstration of the need for Spi-1 to bind DNA to favor the use of the proximal 5′ alternative splice site. The increase in transcription as well as the splicing effect of Spi-1 on E1A RNA transcribed from the CMV promoter was less significant than the effects on E1A RNA whose transcription was driven by the fes promoter (9-fold transactivation and 60% of the 9 S proportion found in the control cells, Fig. 1, B and C). We decided not to pursue the experiments using the CMV promoter because multiple transcription factors bind to the CMV promoter and most probably attenuate the Spi-1 effects, impeding a proper interpretation of the data.

In conclusion, when the Spi-1 protein recognizes responsive elements in the transcriptional promoter and transactivates, it favors the use of the proximal 5′ alternative splice site. Conversely, when Spi-1 cannot bind DNA responsive elements, this qualitative splicing effect is lost.

The Effect of Spi-1 on Splicing Is Independent of Transcription Levels—The experiments presented above suggest that the role played by Spi-1 in transcription and splicing is co-regulated. So, it was of interest to determine whether the modifications of splicing profiles in the presence of Spi-1 resulted from differences in the abundance of pre-mRNA. To examine this question, we wondered whether the number of transcriptional units affects the processing of the pre-mRNAs by Spi-1.

First, we compared the splicing patterns of E1A pre-mRNA transcribed from the same quantity of vector carrying promoters of different strengths (Figs. 1 and 2). Similar patterns of E1A splicing isoforms were obtained with the fes and fli-1 promoters in the absence of Spi-1 even though the basal level of the E1A transcript was 3 times higher when controlled by the fes promoter compared with the fli-1 promoter (3,700 relative light units for the fes promoter, in the absence of Spi-1, Fig. 1B; and 1,300 relative light units for the fli-1 promoter in the absence of Spi-1, Fig. 2B). Second, 300 ng of Spi-1 protein induced a level of transcription 9-fold higher when driven from the fes promoter than from the fli-1 promoter as illustrated by the differential luciferase activities (54,500 relative light units for the fes promoter, Fig. 1B; and 6,300 relative light units for the fli-1 promoter, Fig. 2B). Nevertheless, Spi-1 favored the use of the 5′ proximal site when the E1A pre-mRNA was expressed downstream from both the fes and fli-1 promoters (Figs. 1C and 2C).
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Subsequently, the splicing patterns for two different quantities of E1A pre-mRNA transcribed from p[fes-wt-E1A-IRES-LucF were analyzed. To be comparable, the total quantities of DNA transfected in the cells have been equalized using the pBlueScript plasmid. As shown in Fig. 6, 12.5 and 100 ng of p[fes-wt-E1A-IRES-LucF transfected in the cells generated different amounts of E1A pre-mRNA as determined from the luciferase activities (in the absence as well as in the presence of Spi-1). Despite the differences in the quantities of pre-mRNA produced from the two quantities of reporter vectors, the maturation of the E1A pre-mRNA was similar regardless of the presence or absence of Spi-1 (Fig. 6).

These results show that the decrease in the relative proportion of 9 S by Spi-1 is independent of the amount of target pre-mRNA expressed or the amount of reporter vector transfected. This result suggests that the augmentation of the number of transcriptional units by Spi-1 does not saturate the splicing machinery. In conclusion, these data are in agreement with the fact that the splicing effects of Spi-1 are not a consequence of differences in the synthesis level of RNA but depend on a qualitative effect of Spi-1 in splicing.

Analysis of the Effect of Spi-1 Mutants on Splicing of E1A Pre-mRNA—To further investigate the relationship between the splicing and transcription functions of Spi-1, we examined the correlation between the ability of Spi-1 to bind DNA, to transactivate and to modulate alternative splicing of E1A using various Spi-1 mutants (Fig. 7A). The Spi-1 mutants were synthesized in reticulocyte lysates and their DNA binding ability was analyzed by an EMSA using the [fes DNA binding site as probe (Fig. 7B). Δ-Pest-Spi-1, devoid of the PEST domain, and DBD-Spi, lacking the transactivation and PEST domains, induced a shift in probe migration (Fig. 7B), consistent with the presence of the DBD in these two mutants. Δ-Cter-Spi-1 lacks the 27 carboxyl-terminal amino acids and Δ-β4-Spi-1 contains deletions of 5 amino acids in the β4 region (39). No protein-DNA complex was detected in EMSA using these mutants, showing that they were deficient in DNA binding (Fig. 7B). Similar results were obtained with the [fli-1 probe (data not shown).

Fig. 8 presents the transcriptional activity of the mutants evaluated in transactivation experiments performed in HeLa cells using p[fes-wt-E1A-IRES-LucF and 20 ng of Spi-1 expression vectors (Fig. 8A) or p[fli-wt-E1A-IRES-LucF and 300 ng of Spi-1 expression vectors (Fig. 8B). Δ-Pest-Spi-1, as the wild-type Spi-1 protein (wt-Spi-1), stimulated luciferase activity as compared with the control, indicating an increased transcription (Fig. 8, upper histograms).
Although able to bind DNA, DBD-Spi did not significantly induce luciferase activity, consistent with the lack of an activation domain. Similarly, Δ-Cter-Spi-1 and Δ-β4-Spi-1 did not activate transcription from fes and fli-1 promoters as deduced from the absence of increased luciferase activity. In terms of splicing activity, Δ-Pest-Spi-1 decreased 9 S RNA production as did the intact Spi-1 protein (Fig. 8, lower histograms). Interestingly, the mutants that did not transactivate whether they bound (DBD-Spi-1) or not (Δ-Cter-Spi-1 and Δ-β4-Spi-1) to the Spi-1 sensitive promoters were not able to exert the splicing activity of the wt-Spi-1 protein. The relative proportion of 13 S, 12 S, and 9 S E1A isoforms were either similar to that observed in cells transfected with the empty vector (DBD-Spi-1) or displayed an increase in the 9 S isoform that was counterbalanced by a reduction of the 13 S isoform (Δ-Cter-Spi-1 and Δ-β4-Spi-1; 26 and 31% or 42 and 48% of 9 S increase compared with control cells for fes promoter and fli-1 promoter, respectively). So, in the absence of binding to DNA and transactivation, such as observed for Δ-Cter-Spi-1 and Δ-β4-Spi-1, the splicing patterns revealed a shift toward a processing using the distal 5' site. This splicing profile was reminiscent of the effect of wt-Spi-1 on E1A transcribed from the fes-mut promoter. It is interesting to note that the mutant effects of Spi-1 on pre-mRNA processing were similar when transcription was conducted from fes or fli-1 promoters. These results are again consistent with the idea that Spi-1 favors the use of the proximal 5’ alternative splice site only when bound to DNA and able to transactivate.

FIGURE 6. Splicing effect of Spi-1 is not a consequence of a change in the transcriptional level. HeLa cells were co-transfected with 20 ng of Spi-1 vector, 12.5 or 100 ng of pBBS-wt-E1A-IRE-Luc, and 10 ng of the pCMV-LucR normalization vector. The mean ± S.D. of three independent experiments with duplicate samples are shown. A, effect of Spi-1 protein expression on transcription as a function of different quantities of E1A expression plasmid used. The histograms represent the relative light units normalized to the transfection efficiency; the exact value of the relative light units is indicated above each histogram. B, histograms representing the relative percentage of 13 S, 12 S, and 9 S mRNA isoforms detected are shown and the %9S decrease compared with control cells is indicated.

DISCUSSION

We have previously shown that the oncogenic protein Spi-1/PU.1, initially described as a transcription factor, also affects splicing (22, 23, 25). The splicing function requires the DNA binding domain containing the ETS region (24). This led to the hypothesis that Spi-1 may affect the splicing of a gene whose transcription it regulates. Thus, we developed a model system allowing the characterization of pre-mRNA transcription and maturation from a single minigene whose transcription is driven by Spi-1-dependent promoters. Here, we demonstrate that co-regulation of transcription and splicing can take place through the promoter-specific loading of Spi-1. Furthermore, our results establish that Spi-1 splicing activity is dependent on its DNA binding ability, which is associated to its transactivation effect. The absence of a correlation between promoter strength or number of transcripts and splicing modulation indicates that the function of Spi-1 in splicing is not simply due to its modulation of transcription but is related to the intrinsic properties of the Spi-1 protein. This is consistent with previously published data (27, 28, 33, 34, 40).

The splicing activity of Spi-1 has been previously characterized using E1A pre-mRNA whose transcription was driven by the CMV promoter (22, 23, 25). Spi-1 inhibited the 9 S E1A production as observed for the fes-wt and fli-1-wt promoters. The data presented here show that Spi-1 is able to bind and transactivate the CMV promoter. Altogether, these data demonstrate that Spi-1 exhibits a coordinated action in transcription and splicing.

The observation that promoter structure contributes to alternative splicing constituted one of the first mechanistic data toward the understanding of how alternative splicing and transcription are co-regulated (27). Since then, several studies have
shown that this process is complex and involves cell-specific promoter occupancy associated with splicing factors activity (for review, Ref. 26). Actually, proteins of the spliceosome interacting physically with the transcriptional machinery or proteins playing a role directly in both mechanisms have been proposed to be candidates linking transcription and splicing (26, 30–35, 41). According to the cell-specific promoter occupation model, Spi-1, by recruiting splicing proteins to specific promoters, would play the role of scaffold to bring splicing proteins to the nascent transcribed RNA. Currently, no splicing factor has been identified that could be recruited by Spi-1 and be a candidate for mediating the Spi-1 splicing effect on a transcriptional target RNA. Indeed, Spi-1 interacts with several proteins participating mediating Spi-1 splicing effects when it is not bound to DNA. Indeed, the proportion of the 9 S mRNA isoform was augmented due to an increased use of the distal 5' splice site. These results may be explained by the fact that Spi-1, not bound to DNA, traps and modifies the equilibrium of available splicing factors acting on E1A pre-mRNA processes. In this respect, a high level of Spi-1 expression may modify the alternative splicing of a gene whose transcription it does not control, and, in this case, it would act as an interfering protein. Again, even if the Spi-1 interference on TLS, p54nrb, and the polypyrimidine tract-binding protein-associated splicing factor could take place in the absence of Spi-1 binding to the promoter, it would result in an inhibition of the distal alternative 5' splice site, which is contrary to the Spi-1 effect seen on a gene whose transcription it does not control. Proteins interacting with Spi-1 that would modulate RNA splicing may not be the same whether Spi-1 acts together with SR proteins to favor selection of the proximal alternative 5'-splice site.

When Spi-1 was unable to bind DNA, such as for wt-Spi-1 on mutated promoters or Δ-Cter-Spi-1 and Δ-β4-Spi-1 on wt promoters, it was associated with a splicing pattern opposite to the splicing effect of Spi-1 bound to DNA. Therefore, it will be interesting to examine whether Spi-1 acts together with SR proteins to favor selection of the proximal alternative 5'-splice site.

An alternate hypothesis may involve control of the elongation rate of transcription. It has been shown that transcription can also control splicing through regulation of RNA polymerase II processes and elongation rates (40, 45–47). Notably, the Brm subunit of the SWI/SNF complex, involved in chromatin remodeling on promoters, has been recently demonstrated to contribute to cross-talk between transcription and alternative splicing by decreasing the RNA polymerase II elongation rate (48). Interestingly, a high RNA polymerase II elongation rate favors the use of the proximal 5’ splice site of the adenovirus E1A minigene, generating increased amounts of the 13 S isoform and decreasing the 9 S isoform. This would be compatible with the combined activities of Spi-1 in transcription and splicing.
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for Spi-1 in regulating pol II elongation has not so far been described. Consequently, more studies are required to draw definitive conclusions.

Differences in splicing have been noted between normal and tumor cells. These differences are often due to deregulation of splicing factors in tumor cells (49). A subset of aberrant RNA-spliced isoforms may confer a selective advantage to cancer cells, even if some of the modifications of splicing may be associated with stress induced by the disease (49). The high expression of Spi-1 in mice is associated with the development of erythroleukemia due to the blockage of erythroid differentiation. We have recently demonstrated using the E1A minigene model that in leukemic proerythroblasts, the overexpression of spi-1 affects splicing (24), suggesting that this function may directly contribute to oncogenic activity of Spi-1. The results described in this article were obtained using HeLa epithelial cells. Even if Spi-1 exerts an oncogenic activity only in the erythroid lineage, its splicing activity is not restricted to erythroid cells but was also detected in myeloid, T and B lymphoid, and epithelial cells (Ref. 24 and this study). So, if the interference of Spi-1 in splicing is involved in the blockage of erythroid differentiation, one can envision at least two possibilities. One possibility is that the Spi-1 splicing activity modifies the processing of ubiquitously expressed genes. Because Spi-1 regulates transcription of genes from the myeloid and lymphoid lineages, the splicing effect of Spi-1 in this case would be independent of its activity as a transcription factor. The spliced isoforms specifically associated with the presence of a strong Spi-1 expression would code for a protein modifying only the erythroid differentiation program. Another possibility is that Spi-1 affects genes for which splicing is tightly regulated in a differentiation stage-specific manner. Ample evidence exists that alternative splicing is important for erythroid differentiation (50). A recent publication described the existence of an erythroid cell-specific splice variant of the CP2 transcription factor (51). The protein 4.1R, a vital component of red blood cell membrane cytoskeleton, is also encoded by a gene whose splicing is highly regulated during erythroid differentiation (52, 53). Interestingly, Spi-1 has been recently shown to modify erythroid-specific alternative splicing of 4.1R in murine erythroleukemia cells (54). The SR protein SF2/ASF provokes a splicing modification of the 4.1R RNA that is opposite to the one observed in cells overexpressing Spi-1 (54, 55). Whether Spi-1 competes with SF2/ASF to modulate 4.1R maturation remains to be determined.

In conclusion, we had previously shown that transcription factor Spi-1 is involved in the choice of alternative splice sites used on a pre-mRNA. We have now established that Spi-1 is able to display a specific splicing activity on transcriptional targets. Until now, the involvement of the splicing function of Spi-1 in the blockage of erythroid differentiation and its oncogenic activity has not been established. Our results suggest that if the splicing activity of Spi-1 is involved in blockage of differentiation, it could do so by acting on genes whose transcription it regulates. Nevertheless, the oncogenic function of Spi-1 is directly related to its overexpression. So, overexpressed Spi-1 might act as a titrating factor, as described here, and consequently modify indirectly the processing of transcriptionally independent genes. Strategies are currently being developed to unravel the Spi-1 target genes for splicing and their contribution to oncogenesis.

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