Using hybrid minigene experiments, we have investigated the role of the promoter architecture on the regulation of two alternative spliced exons, cystic fibrosis transmembrane regulator (CFTR) exon 9 and fibronectin extra domain-A (EDA). A specific alternative splicing pattern corresponded to each C-terminal domain (CTD) promoter. Promoter-dependent sensitivity to cotransfected regulatory splicing factor SF2/ASF was observed only for the CFTR exon 9, whereas that of the EDB was refractory to promoter-mediated regulation. Deletion in the CFTR minigene of the downstream intronic splicing silencer element binding SF2/ASF abolished the specific promoter-mediated response to this splicing factor. A systematic analysis of the regulatory cis-acting elements showed that in the presence of suboptimal splice sites or by deletion of exonic enhancer elements the promoter-dependent sensitivity to splicing factor-mediated inhibition was lost. However, the basal regulatory effect of each promoter was preserved. The complex relationships between the promoter-dependent sensitivity to SF2 modulated by the exon 9 definition suggest a kinetic model of promoter-dependent alternative splicing regulation that possibly involves differential RNA polymerase II elongation.

In most eukaryotic cells, transcription and pre-mRNA processing (capping, splicing, and cleavage/polyadenylation) are coordinately regulated within the nucleus both in a temporal and spatial fashion (for review see Refs. 1–3). The phosphorylated C-terminal domain (CTD) of RNA polymerase II (pol II) provides key molecular contacts with these mRNA processing reactions throughout transcriptional elongation and termination. For example, transcripts originating from polymerases without a CTD (T7 polymerase and pol III) cannot be spliced or polyadenylated and, indeed, expression of a form of pol II lacking the CTD does not abrogate transcriptional activity but actually depresses pre-mRNA processing (4). The CTD can physically interact with several pre-mRNA processing factors (4–8), including SR proteins (9), and with transcriptional factors that may have a dual role in transcription and splicing regulation.

The connection between transcription and splicing might have important functional implications in vivo, because the promoter-dependent recruitment of regulatory splicing factor and/or changes in RNA pol II elongation and/or its phosphorylation state may influence the subsequent splice site selection (10). EDA transcript can be modified by changes in the promoter region of the gene, using a transient expression system combined with a promoter swapping processing of the alternative splicing fibronectin (11). It has been clearly shown that this effect is not because of the different mRNA levels produced by each promoter but is related to qualitative properties of the promoters. In addition, the sensitivity to overexpressed SR proteins, which induce EDA exon inclusion, depends on the promoter, thus suggesting that the promoter can modulate regulatory splicing factor action through the participation of the CTD (12). An alternative but not exclusive mechanism suggests that promoters may control alternative splicing via the regulation of pol II elongation or processivity (13, 14). In this case the splice site selection would be critically related to the timing of presentation of the splicing regulatory elements contained in the nascent transcripts. The differential behavior of the α-globin promoter compared with the FN promoter on EDA alternative splicing has recently been found to correlate with different transcriptional processivity (13). The higher processive transcription elongation mediated by the α-globin promoter resulted in excess of the EDA exclusion, whereas the FN promoter gave the reverse effect.

Alternative splicing of the cystic fibrosis transmembrane regulator (CFTR) exon 9, in contrast with fibronectin EDA, is inhibited by SR proteins, thus representing an interesting model for the study of the contribution of the promoter architecture on alternative splicing. The exon 9, at an early stage of synthesis of the CFTR transcript, emerges first from the elongating polymerase followed by the downstream intronic splicing silencer (ISS) element in intron 9. CFTR exon 9 recognition is modulated by exonic regulatory elements and splicing signals at the intron-exon boundaries. At the 3'-end of intron 8 the presence of a variable number of dinucleotide TG (from 9 to 13) followed by a T repeat (T5–T9 or -9) generates polymorphic variants in the population. A high number of TG repeats and a low number of T tracts induces exon skipping (15–17). Serine
arginine-rich proteins (SR proteins) and, in particular the splicing factor 2 (SF2/ASF), interact with the ISS and negatively regulate CFTR exon 9 splicing (18).

In this study we have used the CFTR exon 9 and the FN EDB alternative splicing as models to further analyze the interplay between the promoter architecture and alternative splicing. FN EDB was considered because it shares similar but not identical structure with the previously studied FN EDA exon (11). We have analyzed the sensitivity of promoters to overexpressed SF2/ASF, the role of splice site strength, and the splicing regulatory elements in the mediation of the promoter effect on the alternative splicing. Our results indicate that the promoter has no effect on the FN EDB but can affect CFTR exon 9 alternative splicing. This CFTR exon 9-specific effect is mediated by the presence of the ISS silencer elements binding SR proteins. The exon recognition mediated by the splice sites and by the ESE modulates the promoter-dependent sensitivity to SF2/ASF, suggesting a kinetic role of transcription elongation in splicing.

**EXPERIMENTAL PROCEDURES**

*Construction of Hybrid Minigenes*—The 1508-bp PstI-PstI fragment of the a1-globin minigene in pBS KS containing the promoter region of the a1-globin gene along with the entire three exons of the gene was digested with the preparation of the hybrid minigenes. The a1-globin minigene contains a unique BstEII site in the third exon that was used to clone the previously reported BstEII-BstEII cassette containing different versions of the CFTR exon 9 embedded in the fibronectin EDB genomic sequences (18). The sequences of the fibronectin and CMV promoters used have been previously described; the mutant version of the fibronectin promoter contains disruptive point mutations at the −170 cAMP-response (CRE) and −150 CCAAT regulatory elements (11, 19). The 900-bp SphI and HindIII fragments containing the FNWT, FNMut, and CMV promoters until the second exon of the a1-globin were obtained from the original pSVEDA plasmids (11) and subcloned in the BstEII-BstEII cassette containing different versions of the CFTR exon 9 embedded in the fibronectin EDB genomic sequences (18). The sequences of the fibronectin and CMV promoters used have been previously described; the mutant version of the fibronectin promoter contains disruptive point mutations at the −170 cAMP-response (CRE) and −150 CCAAT regulatory elements (11, 19). The 900-bp SphI and HindIII fragments containing the FNWT, FNMut, and CMV promoters until the second exon of the a1-globin were obtained from the original pSVEDA plasmids (11) and subcloned in the BstEII-BstEII cassette containing different versions of the CFTR exon 9 embedded in the fibronectin EDB genomic sequences (18). The sequences of the fibronectin and CMV promoters used have been previously described; the mutant version of the fibronectin promoter contains disruptive point mutations at the −170 cAMP-response (CRE) and −150 CCAAT regulatory elements (11, 19).

*Analysis of the Hybrid Minigene Expression*—We transfected Hep3B cells with the DOTAP reagent with 3 μg of each reported minigene and with the control empty vector pCG (0.5 μg) (20) with different amounts of the splicing factors SF2/ASF codifying plasmid. RNA extraction was performed after 48 h, and the RNA was digested with Dnase-Rnase free. RT-PCR was done as described (18) with one of the two direct primers, a1, 5′-cgatgacgtgtagtagtg-3′ or a2, 5′-cactcactgactgactgactg-3′, and with the reverse primer b2, 5′-taggaagaaggttcatgac-3′. Amplified products were routinely loaded on 1.7% agarose-EBBr gels. For quantification of the PCR reactions, [α-32P]dCTP was included in the PCR reaction mixture, and the products were loaded on 6% native polyacrilamide gel, dried, and exposed to a Cyclone (PerkinElmer). The counts of each splicing band were detected by the number of C/G present in the PCR product sequence. In dose-response experiments splicing inhibitions were calculated as percentages on the basis of the difference between the basal value corresponding to transfection with the control pCG plasmid and those obtained in the presence of different amounts of SF2/ASF.

**RESULTS**

*Exon-specific Alternative Splicing Regulation by the Promoter Architecture*—Differences in pol II promoter structure have been found to modify the processing of the fibronectin EDA alternative spliced transcript (11, 13). To evaluate the specificity of this phenomenon we have analyzed the effect of several promoters on two alternative spliced exons, the FN EDB and the CFTR exon 9. The FN EDB exon is homologous to the EDA, is positively regulated by several SR proteins (21, 22), and contains enhancer elements both in the exon (ESE) and in the intron (ISE) (21–23). On the other hand, CFTR exon 9 inclusion is modulated by multiple and well characterized exononic and intronic cis-acting elements (18, 24) and is inhibited by SR proteins (18, 25). EDB and CFTR exon 9 hybrid minigene constructs were prepared with the transcription driven by a1-globin, FNWT, FNMut, CMV, and CF promoters (Fig. 1). These minigenes were transfected in Hep3B cells, and the pattern of splicing was analyzed by RT-PCR with the primers a2 and B2, which can detect the inclusion and exclusion of the corresponding alternative spliced exons. Transfection experiments with the CFTR exon 9 showed that the percentage of exon 9 inclusion was dependent on the promoter that drives transcription (Fig. 2A). Inclusion of exon 9 in mature mRNA was predominant with the FNMut promoter (about 90% of exon 9 included) and poor with the a1-globin promoter (about 65% of exon 9 included), whereas intermediate splicing patterns were detected for the other constructs. On the contrary, transfection experiments with the FN EDB minigenes showed a percentage of EDB exon inclusion of about 10% that was not affected by the promoter driving the transcription (Fig. 2B).

These amplification experiments with the primers a2 and B2 were specifically aimed only at detection of the splicing of the alternative spliced exon. To determine whether the effect of the promoters is specific for the alternative spliced exon we analyzed full-length transcripts originating from the pre-mRNA splicing of the entire hybrid minigene. These transcripts, including the two constitutively spliced introns from the a1-globin gene, were detected with primers located in exon 1 and at the FN/exon 3 junction of the a1-globin gene. Fig. 2C shows
that the two full-length transcripts with or without the exons were correctly processed. Once again the percentage of exon 9 inclusion was differentially affected by the promoters, whereas no effect was evident for the EDB exon (data not shown). The different behaviors of the FN EDB and CFTR exon 9 indicate that specific exonic elements, and possibly context determinants, are important in mediating the effect of the promoter in the recognition of alternative spliced exons. Peculiar features of the alternatively spliced CFTR exon 9 may be involved in the promoter-dependent regulation.

**Role of Promoters from Intronless Genes in Pre-mRNA Splicing Processing**—Previous studies have shown that some promoters are not competent regarding pre-mRNA processing. This occurs not only for bacterial T7 RNA polymerase but also for eukaryotic RNA pol III promoters. In addition, the expression of a form of pol II with truncation at the CTD strongly affects pre-mRNA processing (4). We have addressed the question of whether the promoters transcribing intronless genes can be considered functionally competent regarding splicing. If the promoter-mediated regulation of splicing is related to differential loading of splicing factors to the polymerase, we can suppose that there is no reason for intronless promoters to load such regulatory factors. Two pol II promoters that form intronless genes U2 and H4 were cloned in the CFTR exon 9 minigene. Their effect on alternative splicing was analyzed with the primers located in exon 1 and at the FN/exon 3 junction of the α-globin gene, which detect the entire length of the transcript. RT-PCR analysis showed that the U2 and H4 promoters have a normal capacity to splice introns from pre-mRNA transcripts (Fig. 3). The amount of transcript with the inclusion of the exon 9 was similar to the CF promoter. This data indicates that the promoters driving intronless genes contain all the elements required for intron processing.

**The Intronic Splicing Silencer Element Modulates the Promoter-mediated Response to SF2/ASF in CFTR Exon 9 Alternative Splicing**—SR proteins can bind directly to specific RNA sequences and to other splicing factors. In addition they can associate, via the CTD, with the transcription machinery. These interactions could mediate promoter regulation of alternative splicing. In the case of the EDA exon, alternative splicing was shown to be critically dependent on the interaction of SF2/ASF with an ESE that induces exon inclusion (12). With regard to the CFTR exon 9, SF2/ASF requires intact splicing silencer elements (ISS and ESS) to induce exon exclusion, the ISS being the main target of SR-protein interaction (18). We have analyzed the promoter-mediated response to SF2/ASF in relation to changes at these splicing silencer elements. For this purpose three representative promoters (α-globin, CF, and FN-Mut) were selected and cloned upstream of two CFTR silencer variants, one consisting of deletion of the ISS element in intron 9 and the other represented by a single exonic nucleotide substitution (see Fig. 1A). This latter adenine to cytosine substitution in position 146 of the exon (A146 → C) occurs in the previously reported ESS element (Ref. 18 and data not shown). The CFTR minigene constructs were cotransfected with the SF2/ASF plasmid and the resulting splicing pattern analyzed by RT-PCR amplification. Fig. 4, lanes 3 and 5 shows that the deletion of both ISS and ESS together and of the ISS alone resulted in a practically complete exon 9 inclusion from mature mRNA, regardless of the promoter driving transcription. In the same CFTR silencer variants, no significant changes in the percentage of exon inclusion were observed after overexpression of SF2/ASF (Fig. 4, lanes 4 and 6). These results indicate that the promoter effect on CFTR exon 9 alternative splicing requires the interaction of SF2/ASF at the ISS element.

**CFTR Exon 9 Sensitivity to SF2/ASF Depends on Promoter Architecture**—We have analyzed the kinetics of the response to SF2/ASF in the CFTR exon 9 and in the EDB exon in order to investigate the role of SR proteins in relation to the promoter-
mediated regulation of splicing. Constructs containing three representative promoters, α-globin, CF, and FNMut, driving transcription of either the CFTR exon 9 or EDB, were cotransfected with increasing amounts of the SF2/ASF plasmid and the resulting splicing pattern analyzed by RT-PCR amplification. In the CFTR exon 9, SF2/ASF induces exon skipping (18). However, the dose-response curves obtained with an increased amount of SF2/ASF showed that the splicing inhibition was strictly dependent on the promoter driving transcription (Fig. 5A). Each promoter showed a characteristic dose-response curve. The strongest response was observed for the α-globin promoter, whereas the FNMut showed a small inhibitory effect mediated by the SR protein. The CF promoter showed an intermediate splicing inhibition. In addition, we have also analyzed the response of the two intronless promoters, U2 and H4. These two promoters responded equally well to the SF2/ASF-mediated inhibition, further indicating the fully competent capacity of these promoters in splicing processivity (data not shown). On the other hand, cotransfection of the EDB minigene constructs with SF2/ASF showed an increase in the EDB+ variant as previously reported (21, 22) (Fig. 5B). However, and in contrast with the CFTR exon 9 system, quantitative analysis of the percentage of the EDB inclusion induced by the splicing factors indicates that the magnitude of the response to SF2/ASF was independent from the promoter driving transcription (Fig. 5B). To exclude the possibility that the ISE element in the EDB intron +1 (which is absent in the CFTR exon 9 minigenes) could be responsible for the promoter-dependent regulation of CFTR exon 9, we have analyzed EDB minigenes without this cis-acting element. As shown in Fig. 5C, the deletion of the ISE element did not affect sensitivity to the splicing factor, thus indicating that the ISE element is not responsible for the different promoter-mediated processing of the CFTR exon 9 splicing. Altogether, these results point to an important role exerted by multiple cis-acting elements located in the CFTR exon 9 and in the nearby flanking introns in mediating the promoter-specific effect on alternative splicing.

Promoter-dependent Sensitivity to SF2/ASF Is Modulated by 5′- and 3′-Splice Site Recognition Efficiency and by Exonic Splicing Enhancer—The promoter architecture may differentially modulate the pol II processivity, which may as a result affect the interactions between the ISS and the SR proteins. The first element found by the elongating polymerase is the exon 9, which is followed by the ISS in intron 9. To modulate the recognition of CFTR exon 9 we have prepared minigene constructs with nucleotide substitutions at the splice sites or with the ESE deleted. We have previously shown that variations at or near the splice sites increase the proportion of CFTR transcripts without exon 9. This occurs by increasing the number of TG tracts or by reducing the T repeats at the 3′-end of intron 8 or with an G to A mutation at the 5′-splice site that introduces the use of a weak cryptic splice site in the exon (26). Recognition of exon 9 was reduced in the TG13-T3 variant, in the G → A mutation at the 5′-SS, and in the ESE deletion and was increased in the T7 variant. Minigene constructs with three promoters (α-globin, CF, and FNMut) were cotransfected alone or with increasing amounts of the SF2/ASF coding plasmid to analyze the basal effect of the promoter and its specific sensitivity to the splicing factor, respectively.

In the absence of SF2/ASF, the percentage of inclusion of exon 9 in mature mRNA was higher for the FNMut promoter, intermediate for the CF, and poor for the α1-globin promoter (Fig. 6). This pattern was independent from the variations introduced at the splice sites or at the ESE, indicating that the basal differences of the promoter are independent from the exon definition. On the contrary, the dose-response study to cotransfected SF2/ASF showed a different sensitivity according not only to the promoter but also to the changes in exon recognition. In contrast to the TG11T5 minigenes analyzed in Fig. 5, the sensitivity to SF2/ASF for the two splice sites variants and
for the ESE deletion was not differentially affected by the promoters (Fig. 7, A–C). In these cases the minigenes responded with similar dose-response curves to the increasing amount of the splicing factors, indicating that in the presence of weak splice sites or when the ESE is deleted the promoter-mediated sensitivity to the splicing factor SF2/ASF is lost. On the contrary, a change in the length on the polymorphic poly-pyrimidinic tract from T5 to T7 gave a different sensitivity to SF2/ASF, according to the promoters (Fig. 7D). These results indicate that exon definition does not affect the basal promoter-specific alternative splicing pattern but the specific sensitivity of the promoter to SF2/ASF.

**DISCUSSION**

In this study we have analyzed the role of the promoter architecture on the regulation of alternative splicing of two exons, the fibronectin EDB and the CFTR exon 9. We show that the promoter structure can modify the splicing pattern of the CFTR exon 9 but not that of the EDB exon. The "structural unit" of the CFTR responsible for this selective promoter effect includes not only the exon 9 itself, together with its regulatory elements, but also the flanking introns.

The CFTR exon 9 represents a particular case of non-evolutionary conserved alternative splicing where the recognition of the exon is weakened by the SF2/ASF interaction at the downstream intronic ISS element (18). In the first instance, we have analyzed the effect of disrupting the ISS element under several promoter contexts with or without overexpression of SF2/ASF. Mutation of the ISS abolished the splicing inhibition induced by SF2/ASF regardless of the kind of promoter (Fig. 4). This indicates that an intact ISS is necessary for the promoter modulation.

We subsequently analyzed the sensitivity to SF2/ASF in the presence of an intact ISS element in relation to the promoter and to the composition of the exonic regulatory elements. Our results show that sensitivity to overexpressed SF2/ASF on the splicing pattern varies with the promoter structure. The highest splicing inhibition was observed for the α-globin promoter and the lowest for the fibronectin promoter. This promoter-dependent sensitivity to SF2/ASF depends, however, on the strength of the splice sites and on the presence of an intact ESE, two critical cis-acting elements involved in exon definition. According to the exon definition model, pairing between the splice sites across an exon, facilitated by the presence of exonic enhancer elements, forms the basic unit of initial recognition by the splicing machinery (27). This model may be particularly important in those genes with large introns. In the presence of weak splice sites or in the case of the deletion of the ESE, the promoters did not differentially affect sensitivity to the splicing factor (Fig. 7, A–C). This strongly suggests that the effect of the promoter on the CFTR exon 9 splicing depends both on the definition of the exon and on the presence of an
intact ISS element. In addition, the promoters maintain a different splicing pattern on the basal level of CFTR inclusion in all the different variants affecting the exon definition (Fig. 6).

Our results provide additional evidence to clarify the mechanisms that could be involved in the coupling of transcription and alternative splicing. Previous studies originally suggested that the promoter can modulate alternative splicing by differentially recruiting splicing factors to an exonic enhancer element (11, 12). In fact, in the EDA exon sensitivity to the cotransfected SR proteins was critically dependent on the promoter driving transcription; the \( ^{\text{H9251}} \)-globin promoter did not respond to SF2/ASF and the FN promoter responded, strongly inducing exon inclusion. However, in the CFTR exon 9 the \( ^{\text{H9251}} \)-globin promoter showed a higher sensitivity to SF2/ASF than the FN promoter. This indicates that the promoter itself is not responsible for recruiting splicing factors to the site of transcription and cannot mediate a different loading capacity of SR proteins to critical \( \text{cis} \)-acting elements (Fig. 5). This is also consistent with the efficient splicing processivity of promoters from intronless U2 and H4 genes (Fig. 3).

Our results reinforce an alternative mechanism of splice site selection mediated by the promoter that takes into account the different timing of presentation of the critical regulatory elements to the elongating polymerase. Experiments in which RNA pol II pause sites affect alternative splicing have suggested a kinetic link between transcription and splicing. This has been shown by delaying the transcription of an essential splicing inhibitory element required for regulation of tropomyosin exon 3 (28). At the early stages of the synthesis of the CFTR transcript, the exon 9 emerges first from the elongating pol II followed by the downstream ISS. Therefore the alternative splicing decision will involve an initial definition of the CFTR exon 9 by splice sites and ESE recognition followed by subsequent silencing mediated by the intronic ISS. Its role could be imagined as that of a “decoy exon” presenting strong binding sites for the SR protein SF2/ASF. A highly processive polymerase would favor the simultaneous presentation of the exon 9 and the ISS, resulting in exon exclusion. If the pol II elongation is slower, the assembly of exon 9 spliceosomal complex will be favored because it does not have to compete with the ISS complex that will be later transcribed. In addition, the promoter influences the sensitivity to cotransfected SF2/ASF only when the emerging exon is better defined (Fig. 7D). All this is consistent with the fact reported earlier that the \( \alpha \)-globin promoter has a higher processivity than the FN promoter and that this promoter causes EDA exon exclusion (13, 29). However, in contrast to the EDA, the sensitivity to SF2/ASF in the CFTR exon 9 is higher than that of the promoter with a higher pol II processivity, i.e. the \( \alpha \)-globin. This difference can

**FIG. 7. Dose-response curves of exon 9 splicing induced by SF2/ASF as a function of the promoter and splice site variations and ESS deletion.** Hybrid minigenes transcribed with different promoters containing mutation at the 5'-splice site (5'-SS G \( \rightarrow \) A, panel A) or variations at the TGmTn repeats (GT13T3 and GT11T7, panel B and D, respectively) or deletion of the ESE (\( \Delta \) ESE, panel C) were transfected in Hep3B cells with the control plasmid pCG (500 ng) or with increasing amounts of SF2/ASF coding plasmid (50, 100, 250, and 500 ng, respectively). The splicing pattern was then analyzed by RT-PCR with \( \alpha \)2 and B2 primers and resolved on 1.7% agarose gels. The percentage of exon 9 inclusion, shown at the bottom of each lane, is the mean of three independent experiments and was calculated as indicated in the legend of Fig 5. The dose-response curves at the right of each panel show the corresponding splicing inhibition induced by SF2/ASF.
be explained by the different targeting of the splicing factor. In fact, in the EDA SF2/ASF directly participates in the exon definition targeting in the exon itself. On the contrary, in CFTR exon 9 this splicing factor, interacting with the ISS element located in the downstream intron, causes exon skipping. In this dynamic and cotranscriptionally splicing decision a higher processivity of the pol II determined by the promoter is associated with a higher sensitivity to SF2/SF2 in the CFTR exon 9 because of the downstream location of the ISS.

Mutations reducing the exon definition of CFTR exon 9 (such as the T5 allele at the end of intron 8) are frequently found in patients with monosymptomatic forms of cystic fibrosis (16). It is expected that the differential use of transcriptional factors at the promoter affecting the pol II processivity might result in variable expression of the pathological splicing.

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