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# mRNA precursor splicing *in vivo*: Sequence requirements determined by deletion analysis of an intervening sequence

**(RNA processing/intron/globin gene)**

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**ABSTRACT** **To define the extent of intervening sequence required for splicing higher eukaryotic mRNA precursors *in vivo*, we constructed deletions within the second intervening sequence of the human $^{G}\gamma$-globin gene that progressively approach the donor or acceptor splice sites. Most of the intervening sequence can be deleted with no effect on splicing. At the donor splice site, 6 bases of intervening sequence are sufficient for accurate and efficient splicing. At the acceptor splice site, 20 bases are sufficient for accurate and efficient splicing, and 16 bases are sufficient for accurate splicing but at a reduced level. However, 15 bases are insufficient for splicing at a significant level. The effect of deletions ending near the acceptor splice site is independent of whether an A-G dinucleotide is introduced into the acceptor splice site region by the deletion.**

mRNA precursor (pre-mRNA) splicing is the highly accurate and efficient process whereby intervening sequences (IVS) are removed from pre-mRNA and the coding sequences are joined (for review, see ref. 1). Comparisons of many higher eukaryotic IVS have revealed sequence similarity only near the splice junctions, and consensus sequences for the donor (5′) and acceptor (3′) splice sites have been compiled (2). The donor consensus sequence is $\overset{C}{A}$-A-G/g-t-$\overset{a}{g}$-a-g-t (capital letters denote exon sequences, lower case letters denote IVS, / denotes exon–IVS boundary, n denotes any nucleotide). The acceptor consensus sequence is less specific, consisting of a pyrimidine-rich region followed by the dinucleotide A-G: $(\overset{c}{t})_n$-n-$\overset{c}{t}$-a-g/G ($n \geq 11$). In higher eukaryotes, the efficiency of splicing appears to be determined principally by the splice consensus sequences themselves, because mutations within these regions can inhibit splicing (3–10), whereas deletions of internal portions of IVS have no deleterious effect on splicing (11–13). In yeast, an additional sequence upstream from the acceptor splice site is also required for splicing, because deletion or mutation of this sequence results in failure to splice the pre-mRNA (14–16). Recently, internal segments of higher eukaryotic IVS have been shown to be involved in the formation of partially cyclic splicing intermediates (17–19).

In this report, we describe the construction of three series of deletions within the second intervening sequence (IVS-2) of the human $^{G}\gamma$-globin gene that progressively approach either the donor or acceptor splice sites, and we define the extent of sequences near the donor and acceptor splice sites required for accurate and efficient splicing *in vivo*.

## MATERIALS AND METHODS

**Construction of Human $^{G}\gamma$-Globin IVS-2 Deletion Plasmids.** Each series of deletions had one common and one variable endpoint. Thus, within each series, the sequences brought nearer to the splice junction by the deletions were identical (Fig. 1). Deletions ending near the IVS-2 donor splice site (ΔH series) were constructed by cleaving the $^{G}\gamma$-globin plasmid pd$\gamma$(20) with *Hin*dIII 86 bases 5′ to the IVS-2 acceptor splice site (see Fig. 1), followed by complete cleavage with *Xho* I or partial cleavage with *Hin*fI or *Bst*NI. The protruding 5′ ends were then filled in and ligated (21). Deletions ending near the IVS-2 acceptor splice site were constructed by cleaving pd$\gamma$ DNA with *Hin*dIII followed by partial degradation with BAL 31 exonuclease. The DNA was then cleaved with *Xba* I (ΔX series) or *Bss*HII (ΔB series), and the ends were filled in and ligated. Restriction fragments

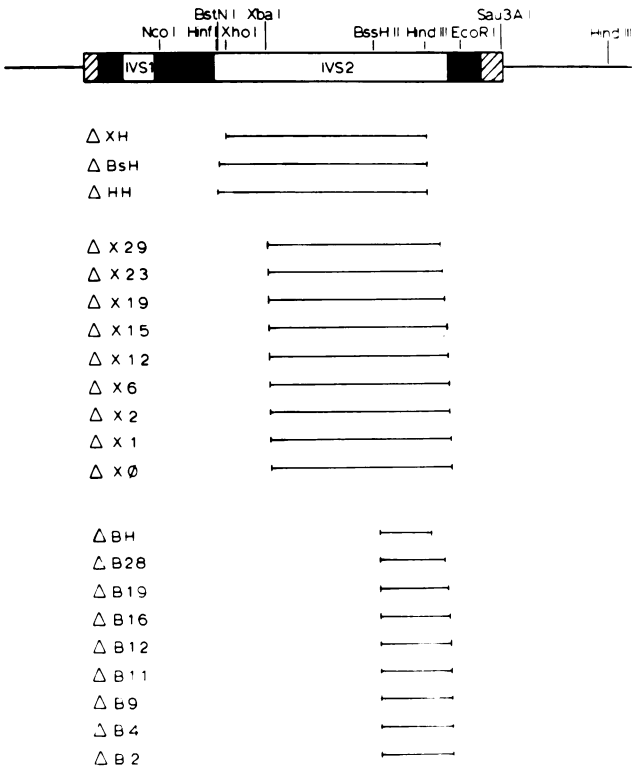


FIG. 1. Structures of $^{G}\gamma$-globin IVS-2 deletion genes. Horizontal bars denote deleted segments. ΔH series deletions end 86 bases 5′ to the acceptor splice site. ΔX series and ΔB series deletions begin 196 and 597 bases 3′ to the donor splice site, respectively. Numbers denote the number of undeleted nucleotides 5′ to the IVS-2 acceptor splice site. In some instances, bases 5′ to the deletion are the same as those deleted. Therefore, ΔB19 and ΔX19 actually have 20 and ΔXØ has 2 bases of identity with sequences immediately 5′ to the normal IVS-2 acceptor splice site. Nucleotide sequences across the deletions are presented in Table 1. Exons are solid; 5′ and 3′ untranslated regions are hatched. Relevant restriction sites are indicated.

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Abbreviations: pre-mRNA, mRNA precursor; bp, base pair(s).

containing the deletions were size-selected on polyacrylamide gels, and the precise extent of each deletion was determined by nucleotide sequencing (22). The normal and deletion γ-globin genes and flanking sequences were all inserted identically into pSVd (20), a simian virus 40 (SV40)–pBR322 "shuttle vector," such that transcription was directed by the γ-globin promoter. Plasmids were propagated in *Escherichia coli* K-12 strain HB101.

**Cell Transfection.** Plasmids were introduced into Cos 7 cells (23) by the DEAE dextran procedure (24), using 2 μg of supercoiled γ-globin plasmid DNA per 100-mm plate. In transfections of ΔX and ΔB series plasmids, an additional 2 μg of a plasmid containing the human $\alpha_1$-globin gene and the SV40 origin of replication [pSVOdα(−); unpublished results] were included to control for transfection efficiency and RNA recovery.

**Preparation and Analysis of RNA.** Total RNA was prepared (25) from transfected cells after 48 hr and fractionated by oligo(dT)-cellulose column chromatography (26). S1 nuclease analyses (27, 28) were performed using 10 μg of polyadenylylated RNA as described (24) with double-stranded end-labeled DNA probes described in the text. S1 nuclease analyses of α-globin transcripts utilized a $^{32}$P 3′-end-labeled 390-base-pair (bp) *Nco* I/*Hin*dIII human $\alpha_1$-globin gene fragment (not shown) as probe.

## RESULTS

**Sequence Requirements at the IVS-2 Donor Splice Site.** The ΔH series IVS-2 deletions with 5′ endpoints near the donor splice site are illustrated in Fig. 1 and in Table 1. ΔXH, ΔBsH, and ΔHH retain 44, 9, and 6 unaltered bases 3′ to the IVS-2 donor splice site, respectively, and each retains 86 bases 5′ to the acceptor splice site.

To measure the extent of correct splicing of transcripts of these deletion genes, we performed S1 nuclease mapping using two $^{32}$P 3′-end-labeled γ-globin probes (Fig. 2). The first probe, a 1007-bp *Nco* I/*Hin*dIII fragment, yields a 203-base fragment when protected by transcripts spliced at the IVS-2 donor splice site. The second probe, a 565-bp *Eco*RI/*Hin*dIII fragment, yields a 166-base protected fragment that serves as a measure of the total amount of correctly terminated polyadenylylated γ-globin transcripts. Both measurements can be made at the same time because the two probes hybridize to nonoverlapping portions of the transcripts. For each ΔH deletion gene, the amount of correctly spliced transcript was approximately equal to that obtained with the undeleted γ-globin gene. The ratio of correctly spliced RNA (203-base band) to total γ-globin RNA (166-base band) was ≈1 in all cases, indicating that splicing at "cryptic" splice sites did not occur to a significant extent. In addition, S1 nuclease analysis demonstrated that ΔXH transcripts were spliced accurately and at a normal level at the IVS-2 acceptor splice site (data not shown). These data demonstrate that most of IVS-2 of the human $^{G}$γ-globin gene can be deleted with no effect on the accuracy or efficiency of splicing, and that 6 bases of IVS-2 at the donor splice site are sufficient to direct normal splicing.

Table 1. Sequences of human $^{G}$γ-globin IVS-2 deletion mutants

| Deletion | Nucleotide sequence | Amount of correctly spliced RNA |
|---|---|---|
| | ΔH series deletions ending near donor splice site | |
| Consensus | ...$^{C}_{A}$AGgt$^{a}_{g}$agt... | |
| Normal | ...CTTCAAGgtgagtccaggagat...19 nt...tagtctcgaggcaac... | ++ |
| ΔXH | ...CTTCAAGgtgagtccaggagat...19 nt...tagtctcgagagctt... | ++ |
| ΔBsH | ...CTTCAAGgtgagtccaagctt ... | ++ |
| ΔHH | ...CTTCAAGgtgagtagctt... | ++ |
| | ΔX series deletions ending near acceptor splice site | |
| Consensus | ...($^{c}_{t}$)$_n$n$^{c}_{t}$agG... | |
| Normal | ...gtggaagct...52 nt...catctttattgtctcctttcatctcaacagCTCCT... | ++ |
| ΔX29 | ...ctgaaaatctagatctttattgtctcctttcatctcaacagCTCCT... | ++ |
| ΔX23 | ...ctgaaaatctagattgtctcctttcatctcaacagCTCCT... | ++ |
| ΔX19 | ...ctgaaaatctagtctcctttcatctcaacagCTCCT... | ++ |
| ΔX15 | ...ctgaaaatctagctttcatctcaacagCTCCT... | Tr |
| ΔX12 | ...ctgaaaatctagtcatctcaacagCTCCT... | Tr |
| ΔX6 | ...ctgaaaatctagcaacagCTCCT... | Tr |
| ΔX2 | ...ctgaaaatctagagCTCCT... | Tr |
| ΔX1 | ...ctgaaaatctaggCTCCT... | Tr |
| ΔXØ | ...ctgaaaatctagCTCCT... | Tr |
| | ΔB series deletions ending near acceptor splice site | |
| ΔBH | ...(gt)$_{11}$gcgcgagct...52 nt...catctttattgtctcctttcatctcaacagCTCCT... | ++ |
| ΔB28 | ...(gt)$_{11}$gcgcgtctttattgtctcctttcatctcaacagCTCCT... | ++ |
| ΔB20 | ...(gt)$_{11}$gcgcggtctcctttcatctcaacagCTCCT... | ++ |
| ΔB19 | ...(gt)$_{11}$gcgcgtctcctttcatctcaacagCTCCT... | + |
| ΔB16 | ...(gt)$_{11}$gcgcgcctttcatctcaacagCTCCT... | + |
| ΔB12 | ...(gt)$_{11}$gcgcgtcatctcaacagCTCCT... | Tr |
| ΔB11 | ...(gt)$_{11}$gcgcgcatctcaacagCTCCT... | Tr |
| ΔB9 | ...(gt)$_{11}$gcgcgtctcaacagCTCCT... | Tr |
| ΔB4 | ...(gt)$_{11}$gcgcgacagCTCCT... | Tr |
| ΔB2 | ...(gt)$_{11}$gcgcgagCTCCT... | Tr |

Capital letters, exon sequences; lower case letters, IVS; bold face, sequences introduced into splice site region by deletions. ++, normal; +, reduced from normal; Tr, trace; n, any nucleotide.

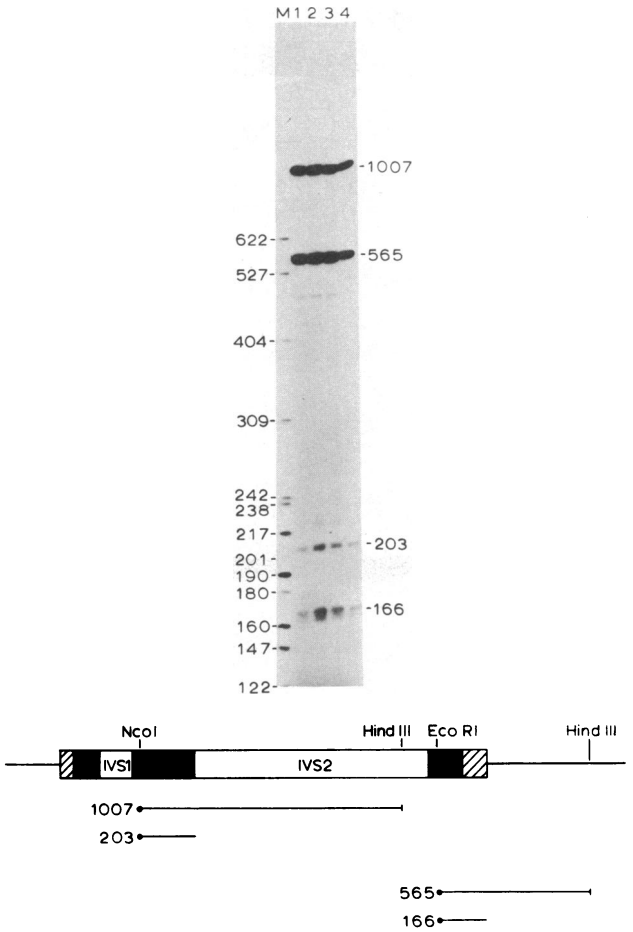


FIG. 2. S1 nuclease analyses of ΔH series deletion transcripts. Probes are described in text. Lanes: M, size markers (bp) (3′-end-labeled *Msp* I digest of pBR322); 1, ΔXH; 2, ΔBsH; 3, ΔHH; 4, normal gene. $^{32}P$ label is denoted by filled circle.

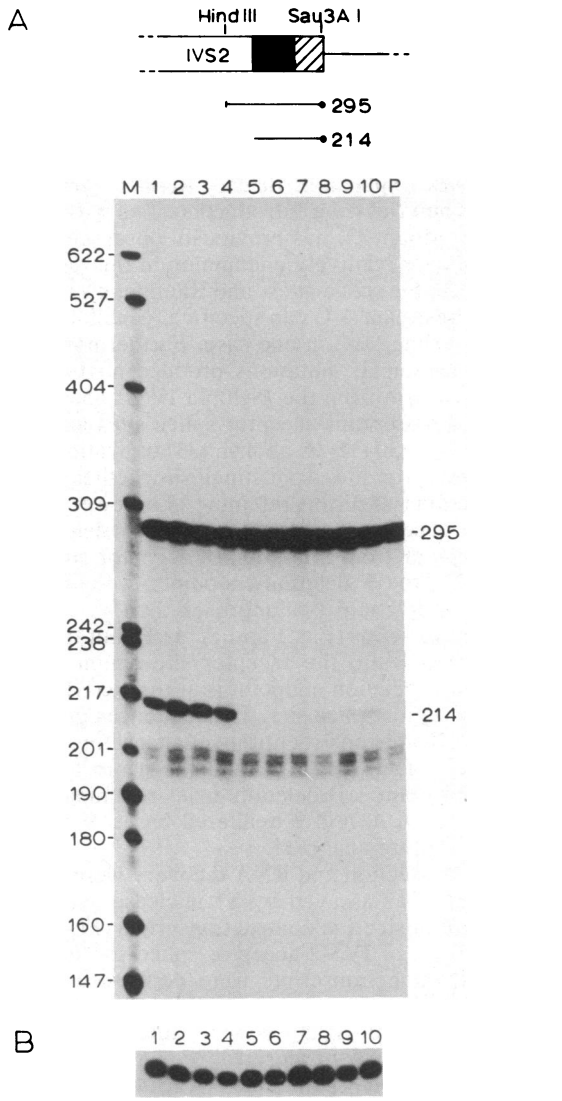


FIG. 3. (*A*) S1 nuclease analyses of ΔX series deletion transcripts. Probe is described in text. Lanes: M, size markers (bp); 1, normal gene; 2, ΔX29; 3, ΔX23; 4, ΔX19; 5, ΔX15; 6, ΔX12; 7, ΔX6; 8, ΔX2; 9, ΔX1; 10, ΔXØ; P, HeLa cytoplasmic RNA. (*B*) S1 nuclease analyses of α-globin cotransfection control transcripts. Probe is described in text. Lanes are the same as in *A*.

**Sequence Requirements at the IVS-2 Acceptor Splice Site.** To define the extent of sequences upstream from the IVS-2 acceptor splice site that are required for splicing, we constructed the ΔX series of IVS-2 deletions that approach the acceptor splice site (Fig. 1 and Table 1). The common 5′ deletion endpoint was the *Xba* I site 196 bases 3′ to the donor splice site, and ΔX series deletion genes retaining 29, 23, 19, 15, 12, 6, 2, 1, and 0 unaltered bases 5′ to the acceptor splice site were analyzed.

Cell transfections and RNA recoveries were comparable for all samples, as determined by S1 nuclease analyses of the α-globin DNA cotransfection control transcripts (Fig. 3*B*). S1 nuclease analyses of IVS-2 acceptor splice site utilization in the ΔX series of deletions were performed using a 295-base $^{32}P$ 5′-end-labeled *Hin*dIII/*Sau*3A I γ-globin probe. γ-globin transcripts correctly spliced at the IVS-2 acceptor splice site protect a 214-base segment of this probe. As shown in Fig. 3*A*, ΔX series deletion genes retaining 19 (ΔX19) or more unaltered bases 5′ to the acceptor splice site produced approximately normal levels of correctly spliced RNA. Deletion genes with 15 (ΔX15) or fewer unaltered bases produced only trace amounts of correctly spliced RNA. We do not know the basis for the three faint bands between 190 and 200 bases. They also appear when no γ-globin RNA is added (Fig. 3*A*, lane P), and they may result from artifactual cleavage by S1 nuclease in A+T-rich regions of the probe.

We also observed trace amounts of RNA that was either unspliced or spliced at a cryptic acceptor site(s) at or 5′ to the deletion. Because homology between the probe (made from undeleted DNA) and the deletion genes ends at the deletion, unspliced transcripts and RNAs spliced at or 5′ to the deletion cannot be distinguished by this analysis. In either case, the RNA would protect a fragment sized 214 bases plus the number of unaltered bases 5′ to the IVS-2 acceptor splice site. The A-G dinucleotide at the 5′ deletion endpoint of the ΔX series of deletions (Table 1) did not itself function as a cryptic acceptor splice site, because at most only trace amounts of aberrantly spliced RNA were detected. Even ΔXØ, in which the A-G at the 5′ deletion endpoint was precisely juxtaposed to exon 3, produced little or no spliced RNA. The amounts of total polyadenylylated ΔX29 and ΔX2 transcripts, assayed by S1 nuclease analysis using the *Eco*RI/*Hin*dIII probe described above (data not shown) corresponded closely to the amounts of correctly spliced RNA, also indicating that cryptic acceptor splice sites were not utilized to any great extent. In addition, S1 nuclease analyses of ΔX29, ΔX12, and ΔX6 transcripts, using the *Nco* I/*Hin*dIII IVS-2 donor site probe described above, demonstrated similar levels of splicing at the normal IVS-2 donor and acceptor

splice sites (data not shown), further indicating that cryptic splice events were uncommon or absent.

These data demonstrate that 20 bases 5′ to the γ-globin IVS-2 acceptor splice site are sufficient to direct accurate and efficient splicing at this site. Fifteen bases are insufficient for splicing at a significant level.

**Decreased Splicing Efficiency Results from Deletion of Sequences and Not from Introduction of an A-G 5′ to the Acceptor Site.** Mount (2) has previously observed that A-G dinucleotides are relatively uncommon in the region 5–25 bases 5′ to acceptor splice sites, and Rautmann *et al.* (29) showed that an upstream A-G can specifically inhibit utilization of an acceptor splice site in one case. Furthermore, in two forms of β-thalassemia, mutations produce novel A-G dinucleotides upstream from the β-globin IVS-1 acceptor splice site that create aberrant acceptor splice sites and also dramatically inhibit (30–32) or abolish (33) utilization of the normal IVS-1 acceptor site. To distinguish whether poor splicing of ΔX deletion transcripts retaining 15 or fewer unaltered bases 5′ to the acceptor splice site results from deletion of required sequences or from inhibition of acceptor site utilization by the A-G at the 5′ deletion endpoints, we constructed a second set of deletions that approach the IVS-2 acceptor splice site, the ΔB series (Fig. 1 and Table 1), but that do not introduce an A-G into the acceptor site region. The ΔB series common 5′ deletion endpoint is at the *Bss*HII site 597 bases 3′ to the donor splice site. The sequences immediately 5′ to the *Bss*HII site consist entirely of alternating purines and pyrimidines [$(T\text{-}G)_{11}$-$(C\text{-}G)_2$], and the nearest A-G occurs 30 bases upstream. ΔB deletion genes retaining 86, 28, 20, 19, 16, 12, 11, 9, 4, and 2 unaltered bases 5′ to the acceptor splice site were analyzed.

Cell transfection and RNA recovery were comparable for all samples, as assayed by S1 nuclease analyses of the α-globin cotransfection control transcripts (Fig. 4*B*). S1 nuclease analyses of IVS-2 acceptor splice site utilization in ΔB series deletion transcripts were performed using the *Hin*dIII/*Sau*3AI probe described above (Fig. 4*A*). ΔB deletion mutants with 20 (ΔB20) or more unaltered bases 5′ to the acceptor splice site produced approximately normal levels of correctly spliced γ-globin mRNA (214-base band). Deletion mutants with 19 (ΔB19) or 16 (ΔB16) unaltered bases produced significantly decreased amounts of correctly spliced mRNA. Deletion mutants with 12 (ΔB12) or fewer unaltered bases produced only trace amounts of correctly spliced mRNA. For each deletion, the amount of correctly spliced RNA corresponded closely to the amount of total polyadenylylated γ-globin RNA (data not shown), again indicating that cryptic IVS-2 acceptor splice sites were not utilized to any great extent. These results are similar to those obtained with the ΔX series of deletions ending near the acceptor splice site. Therefore, we conclude that the sequences introduced into the acceptor splice site region, including an A-G dinucleotide, had no deleterious effect on acceptor site utilization above and beyond the effect of sequence deletion.

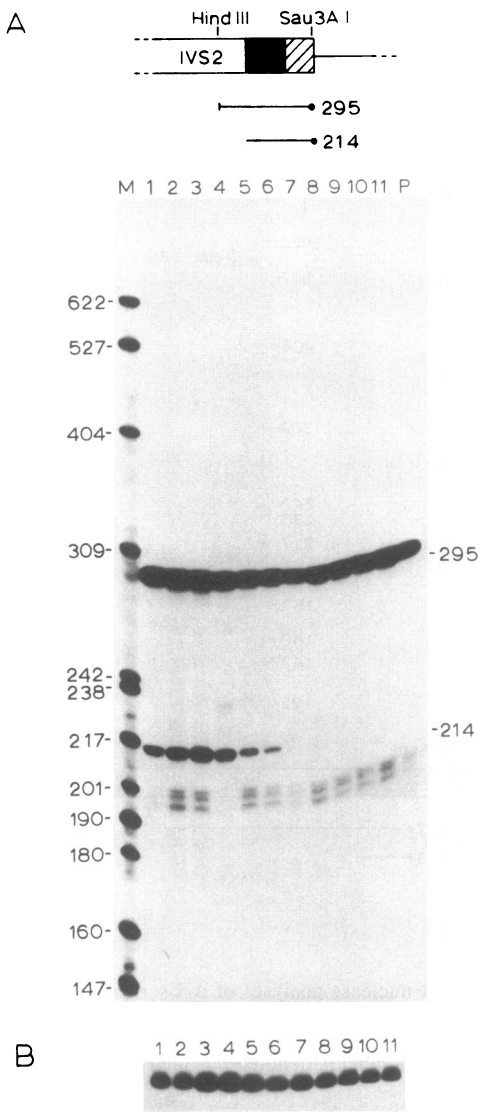


FIG. 4. (*A*) S1 nuclease analyses of ΔB series deletion transcripts. Probe is described in text. Lanes: M, size markers (bp); 1, normal gene; 2, ΔBH; 3, ΔB28; 4, ΔB20; 5, ΔB19; 6, ΔB16; 7, ΔB12; 8, ΔB11; 9, ΔB9; 10, ΔB4; 11, ΔB2; P, HeLa cytoplasmic RNA. (*B*) S1 nuclease analyses of α-globin cotransfection control transcripts. Probe is described in text. Lanes are the same as in *A*.

## DISCUSSION

Using three series of deletions within IVS-2 of the human $^{G}\gamma$-globin gene, we have demonstrated that most of the γ-globin IVS-2 is dispensable without deleterious effect on RNA splicing. This has also been shown for several other higher eukaryotic IVS (11–13, 34). The largest deletion in this study, ΔHH, leaves an IVS of 92 bases. This is considerably larger than any possible minimum IVS size required for splicing, because a naturally occurring *Caenorhabditis elegans unc*-54 myosin heavy chain gene includes an IVS of only 38 nucleotides (35). Because most of IVS-2 is not necessary for splicing, extensive pre-mRNA higher-order structures involving IVS-2 probably do not play a major role in splice site recognition. Alternatively, elements of RNA higher-order structure that are necessary for splicing may be limited to the regions immediately surrounding the splice sites. At the γ-globin IVS-2 donor splice site, 6 bases of IVS are sufficient to direct accurate and efficient splicing *in vivo*. At the acceptor splice site, 17–20 bases of IVS are required for splicing at a normal level and at least 16 bases are required for splicing at an appreciable level; 15 bases are insufficient. These analyses assayed only the steady-state levels of γ-globin mRNA; therefore, we cannot assess the effect of these deletions on the rate of splicing.

The six bases of IVS at the donor splice site that are sufficient for normal splicing correspond precisely to the segment of IVS included in the donor splice site consensus sequence (2). Many instances of mutations at donor splice sites have confirmed the importance of the donor consensus region for splicing (3–10). Mutation of the guanine at position 1 to adenine (3–5) or the thymine at position 2 to guanine (7) abolishes splicing. Mutations at positions 5 or 6 can decrease the efficiency of splicing (4). However, bases 3 or 4 can, in at

least some cases, be altered without effect on the efficiency or accuracy of splicing (5).

Much of the 30-base pyrimidine-rich region upstream from the γ-globin IVS-2 acceptor splice site is not necessary for splicing. Deletion mutants retaining 20 bases of this region produced normal amounts of correctly spliced γ-globin mRNA. It is difficult to account for decreased splicing of ΔB19 transcripts as compared to ΔB20 and ΔX19, because all of these deletion genes actually retain 20 bases of identity with the sequences normally upstream from the IVS-2 acceptor splice site (Fig. 1 and Table 1). It may be that, as the minimum number of bases 5′ to the acceptor site is approached, upstream sequences exert idiosyncratic effects. ΔB16, which retains 16 bases, also produced a reduced but significant amount of correctly spliced mRNA. However, ΔX15, which retains 15 bases, produced almost none. Provided that the A-G upstream from the IVS-2 acceptor site in ΔX15 had no adverse effect on splicing over and above the effect of the deletion, these data suggest that 16 bases upstream from the γ-globin IVS-2 acceptor splice site are sufficient for splicing, albeit at decreased efficiency, but that 15 are not. While this report was in preparation, Wieringa *et al.* (34) reported a deletion study of the rabbit β-globin IVS-2. Their results are generally similar to ours, although they found that a deletion gene retaining 15 bases 5′ to the acceptor splice site produced about one-third normal levels of correctly spliced steady-state mRNA. Furthermore, a deletion leaving only 12 bases 5′ to the acceptor splice site of SV40 T-antigen RNA allowed production of approximately normal levels of T antigen (11, 12). Altogether, these observations indicate that the precise extent of required sequences varies somewhat among acceptor splice sites.

We do not yet know the nature of the required sequences upstream from the acceptor splice site. Recently, partially cyclic ("lariat") splicing intermediates have been described (17–19) in which the 5′ end of the IVS is covalently linked via a 2′–5′ phosphodiester bond to a "branch site" 24–37 bases upstream from the acceptor splice site. The location of the human $^{G}$γ-globin IVS-2 branch site is not yet known. It may be that this site is deleted in the ΔX and ΔB deletion genes that produce little or no spliced mRNA, preventing formation of an IVS-2 lariat splicing intermediate. Alternatively, deletion of the normal branch site may simply lead to use of cryptic branch sites, and the inhibition of splicing by some deletions results from other causes. Furthermore, it might be that an A-G dinucleotide only interferes with normal splicing when it occurs between the branch site and the acceptor site, accounting for the apparent inhibition of acceptor site use by an upstream A-G in some cases (29–33) but not in others (2).

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