Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1184–1188, March 1983
Biochemistry

Abnormal splice in a mutant human β-globin gene not at the site of a mutation

(S1 nuclease mapping/thalassemia)

CARL DOBKIN, ROBERT G. PERGOLIZZI, PAMELA BAHRE, AND ARTHUR BANK

Columbia University, College of Physicians and Surgeons Department of Medicine and Department of Human Genetics and Development, Hammer Health Sciences Center, New York, New York 10032

Communicated by Harold S. Ginsberg, November 22, 1982

ABSTRACT **We have studied the expression of a cloned mutant human β-globin gene in tissue culture cells. The gene, which was previously isolated from the chromosomal DNA of an individual with a low level of normal β-globin expression (β^+-thalassemia), contains five mutations inside the large intervening sequence (IVS2), as well as a silent change in codon 2. This β-thalassemia gene (*thal*) was inserted into a plasmid that is replicated and transcribed in a line of monkey kidney cells in culture. S1 nuclease mapping of the β-globin RNA transcribed from this gene indicates that some of the β-globin RNA is spliced abnormally by using a cryptic 3′ splice sequence normally present in IVS2 but not used in processing the normal β-globin transcript. The cryptic 3′ splice site is not the site of a mutation in the *thal* gene. Because neither the 5′ or 3′ splice junction nor the cryptic site is mutated in this gene, it is most likely that the mutation at position 705 of IVS2, the only nonpolymorphic change in the gene, interferes indirectly with normal processing. These results suggest that certain sequences within IVS must be conserved to prevent abnormal splicing and loss of gene function.**

β-Thalassemia (Thal) is an inherited disorder in which β-globin production is decreased (β^+-Thal) or absent (β^0-Thal). β^+-Thal has been found to be due to mutations that affect either β-globin mRNA splicing (1–4) or transcription initiation (4, 5). β^0-Thal is due to the presence of termination codons (6–8), partial β-globin gene deletion (9, 10), and mutations at splice junctions (11, 12). The gene that we have investigated was isolated from an individual who had β^+-Thal and whose erythrocyte precursor cells contained increased amounts of large β-globin RNA precursors (13). The sequence of the region from 200 base pairs (bp) 5′ to the site of β-globin RNA initiation (cap) to past the polyadenylylation signal on the 3′ side was determined and contained only six changes (14) (Fig. 1). There is a "silent" third-base change at codon 2 and five changes within intervening sequence 2 (IVS2) (14). Only one of the mutations, at nucleotide 705 of IVS2, is not polymorphic.

To determine the role of the mutations in this *thal* gene, we tested its transcription in a tissue culture expression system that involves a plasmid vector, pSVOd, and a line of monkey kidney cells permissive for its replication (15). Analysis of the β-globin RNA by S1 nuclease mapping (16) shows that there is faithful splicing of β-globin RNA in this system; IVS2 is correctly removed from transcripts of the normal human β-globin gene. Transcripts of the mutant *thal* gene, however, are spliced abnormally by using a cryptic 3′ splice sequence in a region of IVS2 that is not mutated.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

*Eco*RI, S1 nuclease, and T4 DNA ligase were purchased from Boehringer Mannheim. All other restriction enzymes and *Escherichia coli* DNA polymerase I (Klenow fragment) were purchased from New England BioLabs, T4 polynucleotide kinase was from P-L Biochemicals, DEAE-dextran was from Pharmacia Fine Chemicals, and [α-^{32}P]dATP and [γ-^{32}P]ATP were from Amersham. Except where specified, all enzymatic reactions were performed according to the supplier's recommendations.

β-Globin pSVOd Plasmid. The 4.4-kilobase (kb) *Pst* I fragment of human chromosomal DNA containing the *thal* gene (14) was ligated into *Pst* I-cleaved pSVOd (15) (see Fig. 2). The analogous pair of recombinant plasmids containing a normal β-globin gene (17) were also constructed.

COS Cells. The COS-7 cell line (18) was propagated in Dulbecco's modification of Eagle medium supplemented with 10% calf serum. For transfection, 1 μg of plasmid DNA was applied in a solution of DEAE-dextran to a dish of approximately 8×10^5 COS-7 cells (19) for 30 min. Forty-eight to 72 hr later the cells were scraped from the plates and harvested by centrifugation.

RNA Isolation. RNA from approximately 5×10^7 cells was isolated by CsCl centrifugation at 30,000 rpm overnight in a Spinco SW 41 rotor (20). The COS cell RNA pellet (≈1.5 mg) was precipitated with NaCl and ethanol.

S1 Nuclease Mapping. COS cell RNA (50 μg) was hybridized with 0.03 pmol of a double-stranded DNA probe of which only the strand complementary to globin mRNA was end labeled (16, 21). The hybrids were digested with S1 nuclease at $5–10 \times 10^3$ units/ml at 30°C for 90 min (P. Dierks, personal communication). The hybrids were then extracted with phenol, precipitated with ethanol, and electrophoresed on a 5% polyacrylamide/0.25% bisacrylamide sequencing gel (22). The total reticulocyte RNA which served as a control in the S1 nuclease mapping was isolated from human adult peripheral blood (23).

S1 Nuclease Probes. The *Bst*NI–*Hae* III probe which extends from nucleotides 416 to 1,301 was isolated from the β-Thal globin pSVOd plasmid by electrophoresis in a 1% low-temperature-gelling agarose (Bio-Rad) gel (24) and 3′-end labeled with [α-^{32}P]dATP and *E. coli* polymerase I (Klenow) (25). The *Bam*HI–*Eco*RI probe which includes nucleotides 482 through 1,398 was isolated from subclones of this fragment of the normal and of the *thal* genes in pBR322 and 5′-end labeled at the *Eco*RI site (22). The *Bam*HI–*Alu* I probe which includes nucleotides 482 through 1,288 was isolated from this subclone of the *thal* gene (above) and 5′-end labeled at the *Alu* I site (22).

Abbreviations: Thal, thalassemia; *thal*, thalassemia gene; bp, base pair(s); IVS, intervening sequence; kb, kilobase(s).

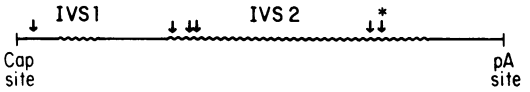

FIG. 1. Mutations in a human β-globin gene from an individual with β^+-Thal. Straight lines, coding sequences; wavy lines, IVS; arrows, positions of the six mutations—C → T in codon 2, 59 bases from the "cap" site; and, numbering from the start of IVS2, C → G at 16, G → T at 74, C → T at 81, T → C at 666, and T → G at 705. The only change that is not a reported polymorphism, at 705, is indicated by an asterisk.

RESULTS

We analyzed the splicing of the β-globin RNA transcribed from the normal and *thal* genes (Fig. 2) in COS cells by S1 nuclease mapping (16).

S1 Nuclease Mapping of the IVS2 5′ Splice Site. We used a *Bst*NI–Hae III fragment of the *thal* gene to determine if the mutations affect the use of the normal IVS2 5′ splice junction. When this probe was hybridized to authentic β-globin mRNA, a single 78-base fragment was protected from S1 nuclease attack (Fig. 3, lanes 3 and 4). This size of fragment marks the use of the normal 5′ splice junction of IVS2 in β-globin RNA processing. Both the normal β-globin gene (lanes 6 and 7) and the *thal* gene (lanes 8 and 9) in pSVOd gave rise to COS cell RNA that protected a probe fragment of this size. Some full-length 886-base probe also was protected by both normal β-globin and Thal COS cell RNA, indicating that some unspliced β-globin transcripts are present. The normal β-globin and Thal lanes are virtually identical. There was no signal between 78 and 886 bases.

S1 Nuclease Mapping of the IVS2 3′ Splice Site. We used a *Bam*HI–*Eco*RI fragment of the β-globin gene to determine if the mutations in IVS2 affect the use of the normal 3′ splice site. When this probe was hybridized to mature β-globin mRNA, a discrete 55-base fragment was protected from S1 nuclease attack (Fig. 4, lanes 2 and 3). This fragment marks the use of the normal 3′ splice site of IVS2 in β-globin RNA processing. Both the normal β-globin (lanes 7 and 8) and the β-Thal (lanes 5 and 6) genes in pSVOd gave rise to COS cell RNA that protected a probe fragment of this size. Some full-length 917-base probe also was protected by normal β-globin and β-Thal COS cell RNA, again indicating the presence of some unspliced transcripts.

There was a striking difference between the normal β-globin

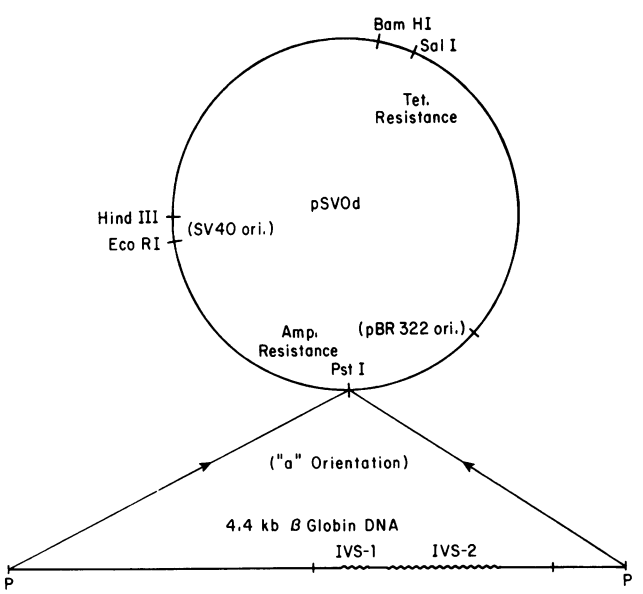

FIG. 2. Insertion of β-globin gene into pSVOd. The 4.4-kb *Pst* I fragment of cloned human chromosomal DNA containing the β-globin gene (14) was inserted into the *Pst* I site of this expression vector, pSVOd (15). The transcribed portion of the gene is shown between the short vertical lines. The orientation of the 4.4-kb fragment in the figure is denoted as "*a*"; the opposite orientation is designated "*b*."

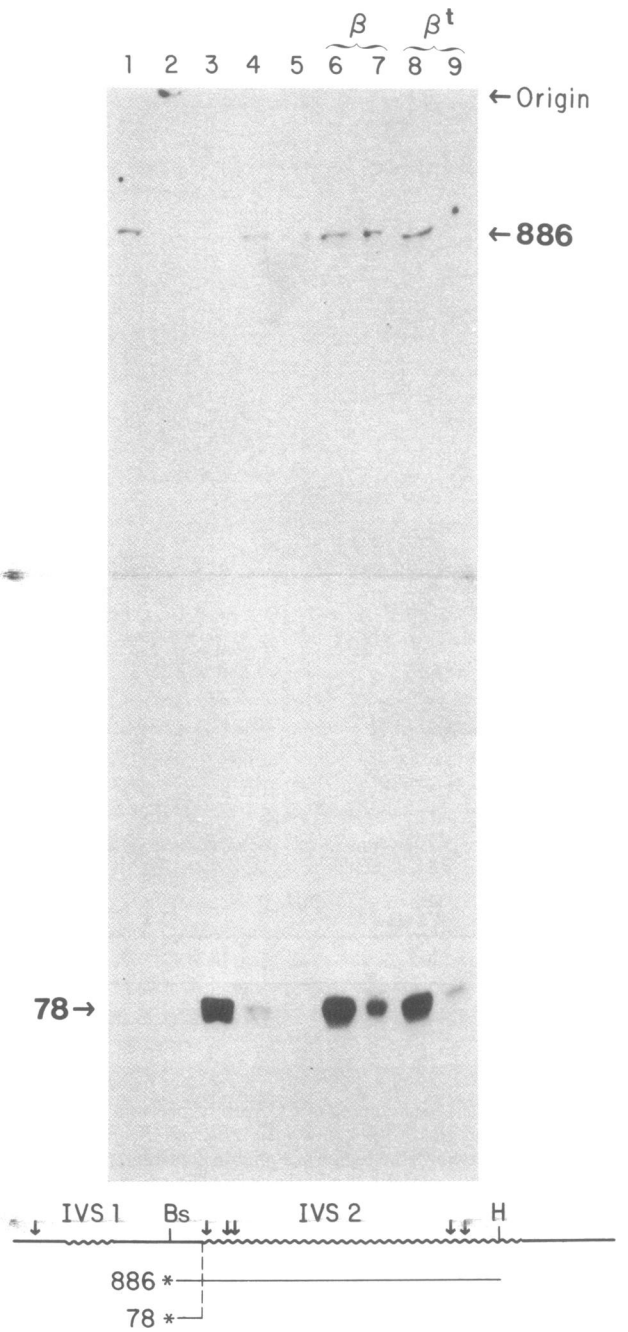

FIG. 3. S1 nuclease mapping of COS cell transcripts of normal β-globin and β-Thal globin (β^t) genes with a *Bst*NI–Hae III probe. The scheme at the bottom shows the region of the β-globin gene represented by the 3′-end labeled (*) *Bst*NI (Bs)–*Hae* III (H) probe. The probe was 886 bases long with 78 bases between the labeled *Bst*NI site and the 5′ border of IVS2. The probe was hybridized to different RNA samples and then treated with S1 nuclease prior to gel electrophoresis. Lanes: 1, undigested probe; 2, no RNA; 3 and 4, 50 and 10 ng of total reticulocyte RNA; 5, RNA from untransfected COS cells; 6 and 7, RNA from COS cells transfected with the *b* and *a* orientations, respectively, of the normal human β-globin gene in pSVOd (see legend to Fig. 2); 8 and 9, RNA from COS cells transfected with *b* and *a* orientations of *thal* gene in pSVOd. The *a* orientation yields fewer transcripts than the *b*. Arrows in scheme at bottom locate *thal* mutations.

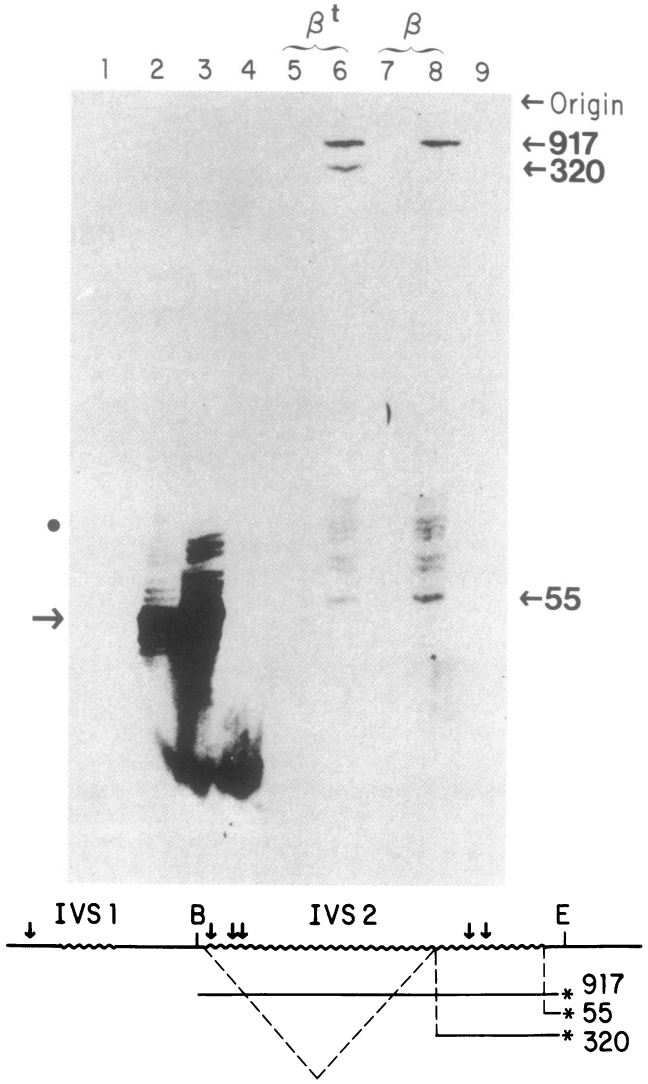

FIG. 4. S1 nuclease mapping of COS cell transcripts of normal β-globin and β-Thal globin (β^t) genes with a *Bam*HI–*Eco*RI probe. The scheme at bottom shows the region of β-globin gene represented by the 5′-end labeled (*) *Bam*HI (B)–*Eco*RI (E) probe. The probe was 917 bases long with 55 bases between the labeled *Eco*RI site and the 3′ border of IVS2. The probe was hybridized to different RNA samples and then treated with S1 nuclease prior to gel electrophoresis. Lanes: 1, *Hpa* II-digested pBR322 size markers (barely visible in this exposure) (dot at left marks 67-base band); 2 and 3, 20 and 100 ng of total reticulocyte RNA [the major band is 55 bases long (arrow at left)]; 4, no RNA; 5 and 6, RNA from COS cells transfected with the *a* and *b* orientations of *thal* in pSVOd (see legend to Fig. 2); 7 and 8, RNA from COS cells transfected with the *a* and *b* orientations of normal β-globin gene; 9, RNA from untransfected COS cells. Upon longer exposure (not shown), the bands in lane 5 were found to correspond to those in lane 6 and the bands in lane 7, to those in lane 8; these results indicate that the orientation of the β-globin gene does not influence the splice products detected, although it does influence the amount of globin RNA transcription. The bands between 55 and 320 bases disappeared with further S1 nuclease digestion. Arrows in the scheme at bottom locate the mutations. The unique 320-base signal in the β-Thal lanes implies a splice to a 3′ site within IVS2 as indicated by dashed line at the bottom.

and β-Thal lanes (Fig. 4, lanes 6 and 8). A new fragment was present in the β-Thal lane between the 917- and 55-base bands. Electrophoresis with markers (not shown) sized the fragment at approximately 320 bases. The presence of this fragment indicates that there is an alternate 3′ splice site which is used in the processing of β-Thal globin transcripts. This new 3′ splice site is at a substantial distance from the five IVS2 mutations (see scheme in Fig. 4). This result implies that the mutations in this gene lead to the use of a cryptic 3′ splice site which is present, but not functional, in the normal β-globin gene.

S1 Nuclease Mapping of the Cryptic 3′ Splice Site. The location of the cryptic 3′ splice site used in the processing of the β-Thal COS cell transcripts was determined more precisely with a *Bam*HI–*Alu* I fragment. A unique 157-base probe fragment was protected by these β-Thal transcripts (Fig. 5). Neither mature β-globin RNA nor transcripts of the normal gene protected a comparable fragment (not shown). Because the *Alu* I site is 170 bases from the *Eco*RI site, this fragment corresponds to the 320-base "cryptic splice" fragment seen with the *Bam*HI–*Eco*RI probe in the previous experiment. When the probe cleaved in the Maxam–Gilbert G and G+A reactions was used as marker, the

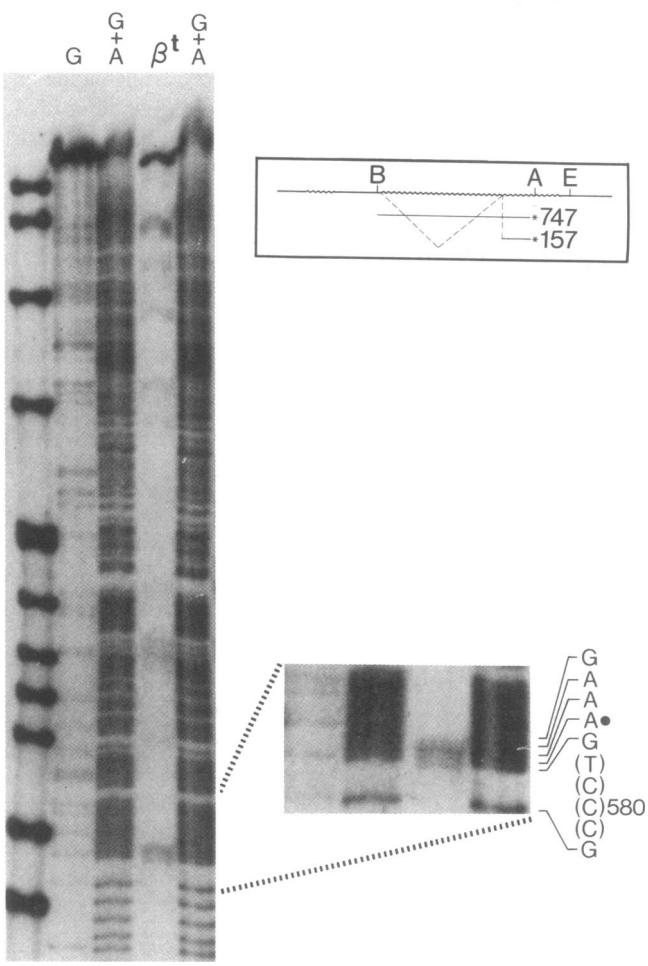

FIG. 5. S1 nuclease mapping of β-Thal (β^t) transcripts with sequence analysis markers. The fragments of the *Bam*HI–*Alu* I probe protected from S1 nuclease digestion by β-Thal RNA were electrophoresed in parallel with the original *Bam*HI–*Alu* I probe cleaved in the Maxam–Gilbert G and G+A reactions. The scheme (upper right corner) shows the region of the β-globin gene represented by the 5′-end labeled (*) *Bam*HI (B)–*Alu* I (A) probe. The probe was 747 bases and the labeled *Alu* I end was within IVS2 170 bases from the *Eco*RI site (E). The 157-base fragment protected only by β-Thal RNA also is shown. Lanes: unlabeled, 5′-end labeled *Hpa* II-digested pBR322; G and G+A, probe cleaved in these Maxam–Gilbert sequence determination reactions; β^t, S1 nuclease mapping fragments of *Bam*HI–*Alu* I probe protected by β-Thal RNA. The unique β-Thal band is more clearly shown in the third lane of the enlargement at the right. In the right-hand margin is the sequence of this region of the probe (bottom to top is 5′ to 3′ in the probe strand, which is complementary to the transcript). The sequence analysis markers indicate that the unique β-Thal band was a fragment that extended primarily to an adenosine (identified by a dot in the margin) 157 bases (±1) from the labeled *Alu* I end.

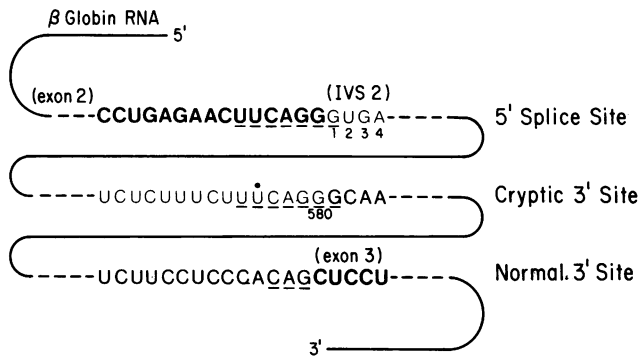

FIG. 6. Sites used in splicing of IVS2 of the β-Thal RNA transcript. The first line contains the sequence of the normal 5′ splice junction, the second is the sequence of the cryptic 3′ site used in the unique β-Thal RNA, and the third is the normal 3′ splice junction. **Boldface,** coding regions 2 and 3; light face, IVS2 (the part of IVS2 retained in the unique β-Thal RNA is shown slightly bolder than the portion removed); underlined letters, nucleotides repeated at 5′ and 3′ splice sites. IVS2 nucleotides are numbered as indicated in first line. Invariant dinucleotides are at positions 1 and 2 (G-T), 578 and 579 (A-G), and 849 and 850 (A-G). Dot over nucleotide 576 marks apparent end (±1) of homology between the *Bam*HI–*Alu* I probe and unique β-Thal RNA (see Fig. 5). This indicates that the splice between the normal 5′ and cryptic 3′ sites retains the repeated septamer sequence (underlined).

fragment protected by the β-Thal COS cell RNA was found to extend to an adenosine residue 157 bases from the labeled *Alu* I end (Fig. 5). This adenosine was 576 bases from the 5′ end of IVS2 in a region that contained not only the A-G dinucleotide present in all 3′ splice sites (26) but also was similar to the 15-base pyrimidine-rich 3′ splice site consensus sequence (27) (Fig. 6). Because the S1 nuclease mapping implies that the homology between the *Alu* I probe and the unique β-Thal RNA extends from the *Alu* I site to nucleotide 576, the splicing presumably occurs near the A-G dinucleotide at 579 in a way that retains the U-U-C-A-G-G-G septamer in the spliced RNA and extends the homology of the probe past the splice point.

DISCUSSION

Using the pSVOd–COS cell system, we have found that the mutations in this β-Thal globin gene lead to aberrant β-globin RNA processing. These mutations result in a splicing event in which the normal 5′ splice junction of IVS2 is joined to a cryptic 3′ site in the middle of the IVS. Surprisingly, neither the normal 5′ or 3′ splice junction nor the cryptic 3′ splice site is near any of the five IVS2 mutations. As indicated earlier, the cryptic site is an excellent 3′ splice site consensus sequence (27) of the form (C or T)$_{11}$ CAG/GG.

How could the mutations in IVS2 in this gene result in the use of a cryptic 3′ splice site that is ignored in the normal gene? Studies with site-directed mutants of the rabbit β-globin gene (28) and with natural mutants of the human gene (12) indicate that, when a splice site is blocked by mutation of the invariant dinucleotide, the splice is completed by use of a nearby cryptic site. There is also a naturally occurring point mutation of the human β-globin gene that creates a new 3′ splice site within IVS1 (3), which is apparently preferred over the normal 3′ splice junction.

We can only speculate on the precise mechanism by which the mutations in *thal* described in this paper lead to the extensive use of the cryptic 3′ site. Of the five IVS2 mutations in this gene, only the one nearest the 3′ end (at IVS2 nucleotide 705) is not polymorphic. We presume that this mutation, either alone or in conjunction with the polymorphisms, is responsible for the aberrant splicing we observe. The change at 705, a T-to-G mutation, creates the sequence G-A-G-G-T-A-A-G-A which looks precisely like a 5′ splice site (27). S1 nuclease mapping with the *Bst*NI–*Hae* III fragment indicates that a splice made by using 705 as a 5′ splice site does not occur alone. In contrast, the data from the *Bam*HI–*Eco*RI probe show that the splice between the normal 5′ junction and the cryptic 3′ site at 580 does occur in the absence of a splice involving the sequence at 705.

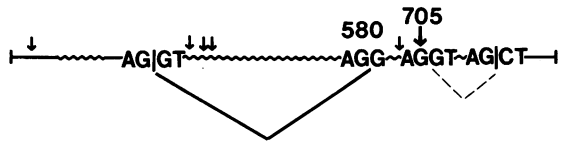

FIG. 7. Splicing of IVS2 in the β-Thal gene. Arrows locate mutations in *thal* gene; the larger arrow locates the nonpolymorphic T → G mutation at nucleotide 705 of IVS2. Solid line, unique β-Thal splice between the normal 5′ junction and the cryptic 3′ site; dashed line, splice between the mutant 5′ splice site at 705 and the normal 3′ splice junction. The sequence at 580 (A-G-G), the cryptic 3′ splice site, is shown. The nucleotide sequence in this region is shown more extensively in Fig. 6. The splice between position 705 and the 3′ splice junction is discussed in the text.

How could an apparently unused 5′ splice site at 705 lead to the use of the cryptic 3′ site at 580? It might do so by competing with the normal 5′ splice junction. If 705 were recognized as a 5′ splice site and formed a potential splice pair with the normal 3′ splice junction, it might prevent the normal 3′ site from being available for normal splicing. This could allow the normal 5′ splice junction and the cryptic 3′ site at 580 to be spliced by default even though the splice involving 705 and the 3′ splice junction were not completed.

Our S1 nuclease mapping results cannot exclude the possibility that the sequence at 705 is used as an additional 5′ splice site in some of the transcripts in which the region from the normal 5′ splice junction to position 580 has already been removed. Preliminary "primer extension" studies with a DNA primer beginning at the *Eco*RI site indicate that the new A-G-G-T sequence at position 705 is used as a 5′ splice site. We find primer extension products in which the region from position 705 to the 3′ end of IVS2 as well as that from the 5′ end of IVS2 to position 580 are absent (Fig. 7). In addition, in these studies we did not detect any transcripts of this *thal* from which all IVS2 had been removed. These results strongly suggest that this is a β^0-Thal gene despite the presence of intact splice junctions. Thus, in this β^+-Thal individual, the other β-globin allele must be a β^+-Thal gene.

The size of the β-globin RNA synthesized in the pSVOd–COS cell system indicates that most transcripts are not initiated at the β-globin promoter (data not shown). However, the demonstration of normal IVS2 splicing by using the normal β-globin gene and the unique abnormalities with the β-Thal gene strongly suggest that the information we have obtained about β-globin RNA splicing in this system is biologically significant.

In addition to establishing a relationship to the generation of the *thal* genotype, these experiments indicate that mutations within an IVS can lead to the use of a cryptic splice site even though the normal splice junctions and the cryptic splice site are not themselves changed. Thus, conservation of certain sequences within IVS is required to preserve normal splicing. Experiments using site-directed mutagenesis in conjunction with an improved tissue culture expression system should disclose the precise relationships between the changes in IVS2 of this gene and aberrant splicing.

We thank Dr. Saul Silverstein for kindly providing COS-7 cells and for guidance in their use, Dr. Peter Dierks for S1 nuclease instructions, Ms. Una Collins for manuscript preparation, and Mr. John Mack and

Mr. Ed Hajjar for preparing illustrations. This work was made possible by National Institutes of Health Grants AM-25274 and CA-31768 and by New York Community Trust Grant 6-40664. C.D. was a Fellow in Cancer Research of the Damon Runyon–Walter Winchell Cancer Fund, DRG-402-Ft, and was also supported by National Institutes of Health Grant HL-29211; R.G.P. was a Postdoctoral Fellow on National Institutes of Health Grant AM-06384.

1. Spritz, R. A., Jagadeeswaran, P., Choudary, P. V., Biro, P. A., Elder, J. T., deRiel, J. K., Manley, J. L., Gefter, M. L., Forget, B. G. & Weissman, S. M. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 2455–2459.
2. Westaway, D. & Williamson, R. (1981) *Nucleic Acids Res.* **9,** 1777–1788.
3. Busslinger, M., Moschonas, N. & Flavell, R. A. (1981) *Cell* **27,** 289–298.
4. Orkin, S., Kazazian, H. H., Antonarakis, S. E., Goff, S., Boehm, C. D., Sexton, J. P., Waber, P. G. & Gefter, M. L. (1982) *Nature (London)* **296,** 627–631.
5. Poncz, M., Ballantine, M., Solowiejczyk, D., Barak, I., Schwartz, E. & Surrey, S. (1982) *J. Biol. Chem.* **257,** 5994–5996.
6. Temple, G. F., Chang, T. C. & Kan, Y. W. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3047–3051.
7. Chang, T. C. & Kan, Y. W. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2886–2889.
8. Pergolizzi, R., Spritz, R. A., Spence, S. E., Goossens, M., Kan, Y. W. & Bank, A. (1981) *Nucleic Acids Res.* **9,** 7065–7072.
9. Orkin, S. H., Kolodner, R., Michaelson, A. & Husson, R. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 3558–3562.
10. Flavell, R. A., Bernards, R., Kooter, J. M. & deBoer, R. (1979) *Nucleic Acids Res.* **6,** 2749–2760.
11. Baird, M., Driscoll, M. C., Schreiner, H., Sciarratta, G. V., Sansone, G., Niazi, G., Ramirez, F. & Bank, A. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4218–4221.
12. Triesman, R., Proudfoot, N. J., Shander, M. & Maniatis, T. (1982) *Cell* **29,** 903–911.
13. Maquat, L. E., Kinniburgh, A. J., Beach, L. R., Honig, G. R., Lazerson, J., Ershler, W. B. & Ross, J. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 4287–4291.
14. Spence, S. E., Pergolizzi, R. G., Donovan-Peluso, M., Kosche, K. A., Dobkin, C. & Bank, A. (1982) *Nucleic Acids Res.* **10,** 1283–1294.
15. Mellon, P., Parker, V., Gluzman, Y. & Maniatis, T. (1981) *Cell* **27,** 279–288.
16. Weaver, R. F. & Weissmann, C. (1979) *Nucleic Acids Res.* **7,** 1175–1193.
17. Lawn, R. M., Efstratiadis, A., O'Connell, C. & Maniatis, T. (1980) *Cell* **21,** 647–651.
18. Gluzman, Y. (1981) *Cell* **23,** 175–182.
19. Myers, R. M. & Tjian, R. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 6491–6495.
20. Glisin, V., Crkvenjakov, R. & Ryus, C. (1974) *Biochemistry* **13,** 2633–2637.
21. Sharp, P. A., Berk, A. J. & Berget, S. M. (1980) *Methods Enzymol.* **65,** 750–768.
22. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–560.
23. Cann, A., Gambino, R., Banks, J. & Bank, A. (1974) *J. Biol. Chem.* **249,** 7536–7540.
24. Weislander, L. (1979) *Anal. Biochem.* **98,** 305–309.
25. Schwartz, E., Scherer, G., Hobon, C. & Kossel, H. (1978) *Nature (London)* **272,** 410–414.
26. Breathnach, R., Benoist, C., O'Hare, K., Gannon, F. & Chambon, P. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4853–4857.
27. Mount, S. M. (1982) *Nucleic Acids Res.* **10,** 459–472.
28. Wieringa, B., Meyer, F., Reiser, J. & Weissmann, C. (1983) *Nature (London)* **301,** 38–43.