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ABNORMAL PROCESSING OF β-MALAY GLOBIN RNA

J.M. Gonzalez-Redondo[1]*, H.E. Brickner[2], and G.F. Atweh[2]

[1] Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, GA 30912-2100

[2] Hematology/Oncology Section, VA Medical Center and Health Science Center at State University of New York, Brooklyn, N.Y. 11209

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Hemoglobin Malay ($\alpha_2\beta_2$19Asn→Ser) has been observed in a few Malaysian patients with thalassemia intermedia. The β^{Malay} substitution increases the homology of the cryptic splice site at codons 17/18/19 of the β-globin gene to the donor consensus splice sequence, suggesting that the β-thalassemia associated with this mutation may be due to the generation of a new splice site. To test this hypothesis, we constructed a hybrid gene where we replaced part of a normal β-globin gene with a PCR amplified region of the β^{Malay} gene. The expression of this mutant gene was studied in a heterologous transient expression system. The data show that nearly 25% of globin mRNA produced by this gene is abnormally spliced at the new splice site, providing a molecular mechanism for the β-thalassemia associated with the mutation.

The β-thalassemias are disorders which result from a decrease in the synthesis of β-globin chains, and are the result of more than 50 different molecular defects (1). Some of these defects are associated with abnormal hemoglobins; these variants are known as thalassemic hemoglobinopathies (2). For some, the thalassemia is caused by the instability of the abnormal β-globin chains while in others (Hb E, Hb Knossos) it is caused by abnormal processing of the RNA (reviewed in Bunn and Forget 1986) (3).

We recently reported the discovery of a new Hb variant, Hb Malay ($\alpha_2\beta_2$19Asn→Ser) in some Malaysian patients with thalassemia intermedia (4). The β^{Malay} mutation, AAC→AGC at codon 19 of the β-globin gene, increases the homology of the sequence located at codons 17/18/19 to the consensus splice sequence (5,6,7), suggesting that the thalassemic features associated with this variant might be due to abnormal RNA processing. In order to investigate this possibility, we undertook studies of the expression of this abnormal globin gene in HeLa cells.

*To whom correspondence should be addressed.

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MATERIAL AND METHODS

Direct cloning of PCR amplified DNA. The genomic DNA of patient B1 (4) was amplified by the PCR method (8) using previously described primers (set A) and amplification conditions (9,10). An ≃700 bp fragment that includes almost 200 nts upstream of the initiation codon, exon 1, intron 1, exon 2, and a small part of the second intron of the β-globin gene was amplified and digested with the restriction enzymes Nco I and Bam HI. An Nco I/Bam HI fragment of a normal β-globin gene cloned into the plasmid pUC19 (11) was removed and replaced with the corresponding fragment of the amplified DNA (10,12) (Fig. 1). This fragment includes the first exon, where the β^{Malay} substitution occurs, the first intron and most of the second exon. Clones positive for the Hb Malay mutation were detected with specific oligonucleotides (4) and the sequence was confirmed by the dideoxy sequencing method of Sanger et al. (13). The β^{Malay} gene was transferred as a Pst I fragment (Fig. 1) from the pUC19 plasmid to the Pst I site of the expression vector πSVplac (5,14).

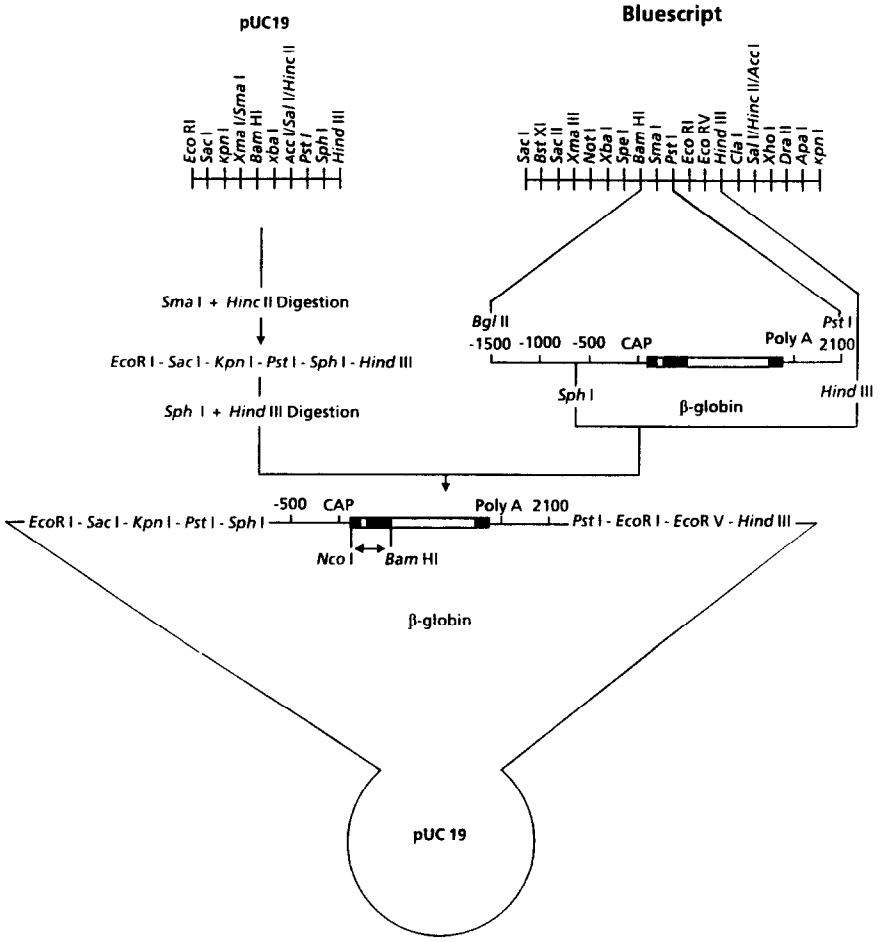

Figure 1. Cloning of amplified DNA into the plasmid pUC19. The plasmid pUC19 was digested with the restriction enzymes Sma I and Hinc II to remove the Bam HI site, religated, and a normal β-globin gene, subcloned in the vector Bluescript (Stratagene, La Jolla, CA) as a Bgl II/Pst I fragment was inserted in pUC19 after digestion of both plasmids with Sph I and Hind III. The Nco I/Bam HI fragment of this new plasmid was replaced by the corresponding fragment obtained by PCR amplification of genomic DNA of patient B1, resulting in a complete β-globin gene.

Gene Expression Studies: HeLa cells were transfected with the constructs of interest, glycerol-shocked and total RNA was harvested exactly as described (6). As a control for the efficiency of transfection, the experimental constructs were cotransfected into HeLa cells along with a construct containing the human γ-globin gene. The mRNA processing of the β^{Malay} hybrid gene was compared to that of a normal β-globin gene and a β-thalassemic globin gene carrying a mutation at IVS-I-5 G→T (6). The latter gene was selected because our previous studies demonstrated the utilization of the cryptic splice site at codons 17,18,19 in the mutant mRNA. The globin RNA from the transfected cells was mapped by S1 nuclease (6,14,15) using an Nco I/Bam HI 3'-end labeled fragment of the β^{Malay} hybrid gene which extends from the translation initiation codon to the 3' end of exon 2 (Fig. 2).

RESULTS

About half of the colonies obtained in the initial cloning of the amplified DNA in the vector pUC19 hybridized to the Hb Malay mutant oligonucleotide. The plasmid obtained from one positive colony was sequenced from the Nco I to the Bam HI site in the β-globin gene, confirming the presence of the mutation. The cloning of DNA fragments by the procedure described above may give rise to artifactual mutations, making sequencing of the clones necessary (17,18). In this case, however, the sequence of the first clone we selected was identical to the published sequence of a normal β-globin gene except at the site of the Malay mutation.

The results of the S1 nuclease mapping experiment are shown in Fig. 2. β-globin mRNA from HeLa cells transfected with a normal β-globin gene protects a 92 nt fragment which extends from the translation initiation codon to the normal exon 1/intron 1 junction (Lane 1). β-globin mRNA from cells transfected with the IVS-1-5 G→T mutant globin gene protects the same 3 different fragments that were previously described (6). These fragments correspond to the utilization of the normal exon 1/intron 1 junction (92 nt), the cryptic splice site at codons 24/25/26/27 (76 nt) and the cryptic splice site at codons 17/18/19 (54 nt) (Lane 2). Finally, β-globin mRNA from cells transfected with the β^{Malay} mutant gene protects a 92 and a 54 nucleotides fragment which corresponds to the utilization of the normal and codons 17/18/19 splice sites (Lane 3). Densitometric scanning demonstrated that approximately 75% of the β-globin mRNA from this gene was spliced normally and 25% was aberrantly spliced. The amount of accumulated γ-globin mRNA in the transfected cells was comparable (data not shown) indicating a comparable efficiency of transfection.

DISCUSSION

The S1 nuclease mapping data show that in HeLa cells, the β^{Malay} mutation leads to the utilization of the cryptic site located at codons 17/18/19 in about one fourth of the processed globin mRNA molecules. These findings explain the thalassemic phenotype found in the patients. Similar studies of the $\beta^{Knossos}$ and β^{E} genes gave considerably lower values (5 to 8%) of abnormally processed RNA when transfected in HeLa cells (19,20), suggesting that the thalassemia produced by the β^{Malay} substitution is probably more severe than that produced by Hb Knossos or Hb

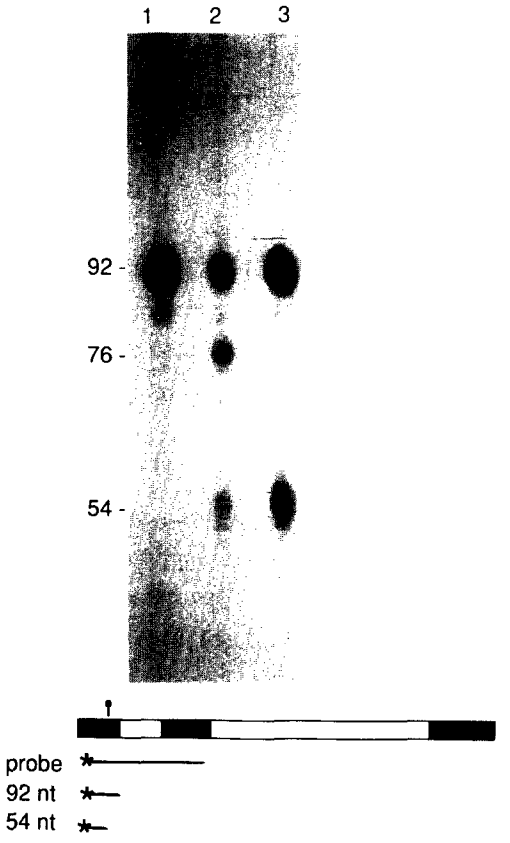

Figure 2. S1 nuclease analysis of the globin mRNA from the different transfection experiments. The origin of the probe used in the experiment is shown on the schematic below the autoradiograph where the dumbell denotes the β^{Malay} mutation site. Lane 1 contains normal β-globin mRNA, Lane 2 contains β-globin mRNA from the IVS-I-5 G→T thalassemic gene and Lane 3 contains mRNA from the β^{Malay} hybrid gene.

E. This assumption is supported by the hematological findings; one of the patients, compound heterozygous for Hb E and Hb Malay, had a significantly lower concentration of β^{Malay} (43.5%) than that of β^{E} (56.5%) chains. Unfortunately, no data is available for the relative quantities of β^{A} and β^{Malay} chains in simple heterozygotes. However, the two Hb Malay homozygotes described by Yang et al. (4) were significantly more anemic than commonly seen in Hb E homozygotes and both subjects had very marked microcytosis and hypochromia.

While three thalassemic mutations that activate the cryptic splice site located at codons 24/25/26/27 have been described (codon 24 GGT→GGA, Hb E and Hb Knossos) (19,20,21), Hb Malay is the first mutation shown to activate the cryptic site at codons 17/18/19. The β^{Malay} mutation affects position 5 of the consensus sequence and gives rise to a splice site with a single mismatch at position 6 with the donor consensus sequence (Fig. 3). The normal exon 1/intron 1 splice junction differs from the donor consensus sequence at two bases (Fig. 3) and is surrounded by three regions of significant homology to the consensus splice signal. These "cryptic sites"

	-3	-2	-1		1	2	3	4	5	6
CONSENSUS SEQUENCE:	C/A	A	G	/	G	T	A/G	A	G	T
Exon 1/IVS-I	C	A	G	/	G	T	T	G	G	T
	Codon 17				Codon 18			Codon 19		
Normal sequence:	A	A	G	/	G	T	G	A	A	C
Hb Malay	A	A	G	/	G	T	G	A	**G**	C

Figure 3. Comparison of the donor splice junction consensus sequence, the donor splice site at exon 1/intron 1 and the normal and β^{Malay} sequences at codon 17/18/19 of the β-globin gene. The mismatches to the consensus sequence are underlined, and the β^{Malay} mutation is boxed.

are used as alternative splice sites in β-thalassemia genes carrying mutations of the exon1/intron junction (5,6). The mechanism responsible for selecting a splice site from several closely related sequences in close proximity is not well understood. Such selection seems to depend not only on the homology of the site to the consensus sequence but also on the context in which the site is located (22,23).

The three known β-thalassemia mutations at position 5 of the first intron of the β-globin gene (IVS-I-5 G→T, IVS-I-5 G→C and IVS-I-5 G→A) result in a profound decrease in the use of the normal splice site and a severe type of thalassemia (4,5,6,24), while the substitution IVS-I-6 T→C produces a very mild type of thalassemia (25). Thus, it appears that the nucleotide at position 5 plays a more significant role in splice site selection than the nucleotide at position 6 (26). The nucleotides at position 3 and 4 have not been implicated in any thalassemic disorder and differ from the consensus in the normal Exon I/IVS-I junction. Since the β^{Malay} cryptic site matches the G consensus at position 5, it may be expected to give rise to a splice site very capable of competing with the normal exon 1/intron 1 splice junction (27). However, the majority of the globin mRNA from this gene is normally spliced and only 25% is aberrantly spliced. This is probably due to more favorable surrounding sequences which contribute to a more efficient use of the exon 1/intron 1 donor splice site. Fortunately, the end result is a relatively mild thalassemic disorder well within the "thalassemia intermedia" clinical classification.

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