Expression of a Cloned Lepore Globin Gene

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Lepore globin is synthesized in markedly diminished amounts (~10% to 15% of normal β-globin) in human erythroid cells. To study the molecular mechanisms responsible for the diminished biosynthesis of Lepore globin, the Lepore–Boston gene was cloned from a charon phage DNA library and expressed in HeLa cells. Northern blotting and S1 nuclease analyses indicated that the Lepore gene produced less globin mRNA than a β-gene and more than a δ-gene. The results indicate that expression of the Lepore–Boston gene in HeLa cells is reduced to an extent comparable to that seen in erythroid precursors in vivo. This indicates that the decrease in Lepore globin gene transcription is due to the δ-nucleotide sequences either in the 5′ flanking region or within this gene.

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MATERIALS AND METHODS

A Lepore–Boston gene was cloned from a library of DNA fragments inserted into charon 27 and isolated by one of us (A.M.) from a typical patient (L.P.) homozygous for hemoglobin Lepore–Boston. The PstI fragment containing the Lepore globin gene was subcloned into a plasmid, pBSV, at the BamHI site (Fig 1). To do this, we synthesized (on an Applied Biosystems Synthesizer) an eight-base oligomer, GATCTGCA, which is complementary to the PstI and BamHI overhanging ends. The ligation of this oligomer to the Lepore globin PstI fragment resulted in a fragment that could be inserted into the BamHI site of pBSV (Fig 1). This plasmid contained approximately 0.5 kilobases (kb) of sequence 5′ to the structural Lepore globin gene and ~0.5 kb of 3′ to this gene. In these studies, δ-pBSV was also constructed from a PstI fragment of an isolated δ-gene. The β-pBSV was also constructed, using a BglII fragment containing a normal δ-globin gene that has ~1.5 kb of 5′ flanking sequence and 1.8 kb of 3′ flanking sequence.

HeLa cells were transfected with these plasmids using DEAE-Dextran. RNA was isolated after 48 hours by cesium chloride sedimentation. RNA was analyzed by Northern blotting, and S1 nuclease protection and RNAase protection when an RNA probe was used. Three different probes were used in the analyses: a β-globin gene segment extending from the BalI site 5′ to the gene to the EcoRI site in exon 3, in bacteriophage M13 mp 7, was used as template for synthesis of uniformly labeled probe. A Lepore globin gene fragment extending from the 5′ PstI site to the EcoRI site in coding region 3 was used in the same way. An RNA probe was prepared by inserting a BalI–EcoRI fragment of the β-globin gene “downstream” from the SP6 promoter in the vector pSP62; uniformly labeled RNA was transcribed from this insert by SP6 RNA polymerase using the instructions of the supplier (Promega Biotec or New England Nuclear, Boston). A 2.3-kb KpnI–BamHI SV 40 fragment was “nick translated” to probe Northern blots for T antigen transcripts.

Northern blots were prepared after agarose gel electrophoresis in the presence of formaldehyde and were hybridized with β-globin probe. Uniformly labeled RNA probe at 0.3 Ci/mg was able to detect Lepore transcripts. S1 nuclease mapping was carried out using the Lepore and β-DNA probes. A 32P-labeled RNA probe was used in the analysis of globin transcripts in ribonuclease mapping experiments by hybridization of the cellular RNA with the probe and treatment of the hybrids with ribonucleases A and T1.

RESULTS

RNAs transcribed in HeLa cells from the Lepore, β-, and δ-pBSV constructs were compared. A “nick-translated” β-globin probe was used in initial Northern blotting experiments, which showed a strong signal from the β-pBSV transcripts, but no signal in RNA from HeLa cells transformed with either the Lepore plasmids or the δ-plasmids (data not shown). This implied that δ-globin mRNA accumulates in HeLa cells transfected with this gene at a lesser rate than with a β-gene. The β-pBSV was also constructed, using a BglII fragment containing a normal δ-globin gene that has ~1.5 kb of 5′ flanking sequence and 1.8 kb of 3′ flanking sequence.

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LEPOR E GLOBIN GENE EXPRESSION

Fig 1. Lepore pBSV expression plasmid. The PstI fragment of the cloned Lepore gene was ligated to a synthetic oligomer, GATCTGCA, in order to create a BamHI end, and inserted into the BamHI site of pBSV. The EcoRI site of pBSV had been eliminated. The PstI fragment contains approximately 0.5 kb 5’ and 0.5 kb 3’ to the gene, which is shown cap site to poly A site, left to right. Open rectangles indicate IVS 1 (δ-sequence). Open rectangle with dots indicates IVS 2 (β-sequence). Solid rectangles indicate exon I and part of exon II, which are δ-sequence. Hatched rectangles indicate the remainder of exon II and exon III, which are β-sequence.

d-globin signal, however. To provide an internal control for plasmid transfection and RNA recovery, RNA from cells transfected with β- and Lepore globin gene containing plasmids were assayed for the presence of transcripts from the plasmid T antigen gene. RNA from cells transfected with either Lepore globin plasmids or β-globin plasmids contains similar amounts of SV 40 T antigen RNA (Fig 2B). Thus, the decrease in Lepore globin mRNA as compared with β-globin mRNA in these same RNA samples is not due to differences in transcription or RNA isolation, but to differences in expression of the β-globin genes and the Lepore globin genes. These results indicate that the Lepore globin gene is expressed in HeLa cells at a level less than that of the β-globin gene and greater than that of the δ-globin gene, which is consistent with the expression of these genes in erythroid cells in vivo.

To further investigate the transcription and processing of RNA from these cloned genes, we analyzed them by S1 nuclease mapping. Using a Lepore DNA probe, a signal was seen with RNAs from HeLa cells transfected with β- and Lepore globin genes containing plasmids, however. To provide an internal control for plasmid transfection and RNA recovery, RNA from cells transfected with β- and Lepore globin gene containing plasmids were assayed for the presence of transcripts from the plasmid T antigen gene. RNA from cells transfected with either Lepore globin plasmids or β-globin plasmids contains similar amounts of SV 40 T antigen RNA (Fig 2B). Thus, the decrease in Lepore globin mRNA as compared with β-globin mRNA in these same RNA samples is not due to differences in transcription or RNA isolation, but to differences in expression of the β-globin genes and the Lepore globin genes. These results indicate that the Lepore globin gene is expressed in HeLa cells at a level less than that of the β-globin gene and greater than that of the δ-globin gene, which is consistent with the expression of these genes in erythroid cells in vivo.

When an RNA probe identical to the β-globin DNA probe was hybridized to β-, δ- and Lepore RNAs, and the hybrids were digested with RNase A and T1, essentially the same pattern of signals is seen as with the DNA probe (Fig 4B). Again, the signal was greatest with β-RNA and substantially reduced with Lepore RNA. The signal with Lepore RNA was again greater than with the δ-RNA.

DISCUSSION

A cloned Lepore–Boston globin gene has been expressed in HeLa cells, and its expression compared to cloned normal δ- and β-human globin genes in this system. The results indicate that the Lepore globin gene is intermediate in its expression between that of normal β- and normal δ-genes, similar to the situation that occurs in vivo in human bone marrow. This suggests that the expression of these transfected genes is regulated by sequences contained within the fragments of DNA transfected. The Lepore gene differs from the β-gene by having δ-sequences in the 5’ flanking region as well as in exon I, the small intervening sequence (IVS 1), and exon II. The decreased expression of the Lepore
Fig 3. S1 mapping of HeLa cell transcripts with Lepore probe. RNA from HeLa cells transfected with β-, Lepore, or δ-globin gene plasmids was hybridized to uniformly labeled DNA, the hybrids were digested with S1 nuclease, the probe fragments were separated on 5% polyacrylamide (19:1, acrylamide:bis acrylamide; 7 mol/L urea) and visualized by autoradiography. The probe was synthesized on a single-stranded template representing the PstI–EcoRI segment of the Lepore gene and isolated after PstI and EcoRI digestion. In the scheme at bottom, the probe is represented as nontranscribed region (line); exon I and part of exon II, which are δ-sequence (solid rectangles); IVS 1, which is δ-sequence (open rectangle); part of exon II and exon III, which are β-sequence (hatched rectangle); and IVS 2, which is β-sequence (open rectangle with dots). The derivation of probe fragments protected by the different RNAs is also diagramed at bottom. Fragments protected by δ-RNA are 145 bases (exon I), 225 bases (exon II), and 49 bases (part of exon III); nonhomology between probe and transcript (arrows at bottom) leads to some S1 cleavage of exon II fragment into 160- and 65-base fragments. (See lanes R and δ.) Fragments protected by Lepore RNA are 145 bases (exon I), 225 bases (exon II), and 49 bases (part of exon III). (See lane L.) Fragments protected by δ-RNA are 145 bases (exon I) and 225 bases (exon II); nonhomology in exon III between probe and δ-annotate prevents protection in this region. (See lane δ.) Lane R: protection by 0.020 μg RNA from human reticulocytes (65-base signal is very light). Lane δ: protection by 50 μg RNA from HeLa cells transfected with δ-globin plasmid. Lane H: protection by 50 μg RNA from HeLa cells (no plasmid). Lane L: protection by 100 μg RNA from HeLa cells transfected with Lepore plasmid (49-base signal is very light). Lane δ: protection by 100 μg RNA from HeLa cells transfected with δ-globin plasmid.

A gene constructed in vitro that resembles the Lepore gene also gives markedly decreased expression when transfected into a line of monkey kidney cells.49 (In these experiments, the BamHI 1.8-kb fragment of the δ-gene was ligated to a 3′ β-fragment at the common Bam site within these genes.) Recent experiments in our laboratory indicate that the expression of a δ-globin gene in HeLa cells is not decreased by substitution of a δ-IVS 1 for a β-IVS 1. Because exons I and II of these genes are >90% homologous, these results suggest that the δ 5′ flank sequences of the Lepore gene are responsible for its decreased expression compared with the normal β-globin gene. Other experiments in our laboratory indicate that substitution of δ-IVS 2 for β-IVS 2 leads to markedly reduced expression. Thus, δ-IVS 2 either decreases the expression of a β-globin gene or β-IVS 2 is required for the high-level expression of this gene. The presence of β-IVS 2 in the Lepore globin gene may explain why it is expressed at a higher level than the δ-globin gene. Thus, elements within as well as flanking the δ- and β-globin genes seem to control their activity—the 5′ flanking region and IVS 2. The Lepore gene with a 5′ δ-flanking region, but a normal β-IVS 2, has a higher rate of expression than the normal δ gene. These experiments do not directly demonstrate the precise step in RNA synthesis responsible for decreased Lepore mRNA accumulation. In addition, further experiments are required to determine whether the 5′ flanking regions and IVS of the δ-, β-, and Lepore genes have these effects when the cloned genes are expressed in erythroid cells.

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Fig 4. (A) S1 mapping of HeLa cell transcripts with β-globin DNA probe. RNA from HeLa cells transfected with β-, Lepore, and δ-globin gene plasmids was hybridized to uniformly labeled DNA, the hybrids were digested with S1 nuclease, the probe fragments were separated on 5% polyacrylamide (19:1, acrylamide:bis-acrylamide; 7 mol/L urea) and visualized by autoradiography. The probe was synthesized on a single-stranded template representing the Ball–EcoRI segment of β-globin DNA and isolated after restriction enzyme digestion. In (C) the probe is represented as nontranscribed region (line), exons I, II, and part of III (hatched rectangles), and IVS 1 and 2 (open rectangles with dots). The derivation of probe fragments protected by the different RNAs is also diagramed at bottom. Fragments protected by β-RNA are 145 bases (exon I), 225 bases (exon II), and 49 bases (part of exon III). (See R and β.) Fragments protected by Lepore RNA are 160, 65 (representing parts of the 225-base exon II segment which is cleaved by S1 due to nonhomology between probe and transcript), and 49 bases (part of exon III). (See L.) Fragments protected by δ-RNA are 160 and 65 bases (representing exon II), and the same fragments are seen with Lepore transcripts. (See δ.) Lane P: undigested probe. Lane R: protection by 0.020 μg RNA from human reticulocytes. Lane β: protection by 50 μg RNA from HeLa cells transfected with β-globin plasmid. Lane L: protection by 100 μg RNA from HeLa cells transfected with Lepore plasmid. Lane δ: protection by 100 μg RNA from HeLa cells transfected with δ-globin plasmid. (The 160-base fragment of exon II is barely discernible.) Lane M: HpaII digested pBR 322 end-labeled marker. (B) Ribonuclease mapping of HeLa cell transcripts with RNA probe representing the β-globin gene. RNA from HeLa cells transfected with β-, Lepore, or δ-globin gene plasmids was hybridized to uniformly labeled RNA, the hybrids were digested with ribonucleases A and T1, the probe fragments were separated on 8% polyacrylamide (19:1, acrylamide:bis acrylamide; 7 mol/L urea) and visualized by autoradiography. The probe was synthesized on a Ball–EcoRI β-globin fragment that had been subcloned (with an EcoRI linker) into the EcoRI site "downstream" from the SP6 promoter in pSP62. Plasmid DNA linearized with Aael (New England Biolabs) was used as template for in vitro synthesis with SP6 RNA polymerase (New England Nuclear or Promega Biotec). This probe is essentially the RNA analog of the probe described in (A). The derivation of the probe fragments is also similar, but additional fragments are visible in Lepore (lane 4); nonhomology between probe and transcript yields more discrete fragments than in the S1 mapping with a DNA probe. Arrows at right locate approximately (from top to bottom) 225-, 160-, 145-, and 49-base fragments. Lanes 1 and 2: protection by 0.020 and 0.050 μg RNA from human reticulocytes. Lane 3: protection by 50 μg HeLa RNA (no plasmid). Lane 4: protection by 100 μg RNA from HeLa cells transfected with Lepore plasmid. Lane 5: protection by 100 μg RNA from HeLa cells transfected with δ-globin plasmid. Lanes 6 and 7: protection by 10 and 25 μg RNA from HeLa cells transfected with β-globin plasmid. Lane 8: HpaII digested pBR 322 and labeled marker. In lane 4, the Lepore RNA lane, a discrete 160-base band is seen as expected (see legend to [A]).

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