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USE OF ANTISENSE RNA TO HELP IDENTIFY A GENOMIC CLONE FOR THE 5' REGION OF MOUSE β-GLUCURONIDASE

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It was possible to gauge the inhibition of mouse β-glucuronidase expression by injecting RNA, made from both strands of subclones of a cosmid containing the complete gene, into mouse blastomeres at the four-cell stage. Although our initial screen did not identify the 5' region, we were able to isolate a subclone containing homology to 20 bp coding for N-terminal amino acids of rat and human β-glucuronidase structural genes. Antisense RNA prepared from one strand of the 350 bp Pst I subclone inhibited β-glucuronidase expression by 89% while RNA prepared from the other strand had little effect. The subclone appears to correspond to the 350 bp fragment identified by others as one including the ATG start site of mouse β-glucuronidase.

Since the discovery of its natural occurrence in bacteria (1), antisense RNA has been used in attempts to study the function of eukaryotic genes both in adult tissues (2) and in embryonic systems (3-6). Antisense molecules are thought to prevent the expression of an mRNA by hybridizing either with the heterogeneous nuclear RNA and blocking its normal processing (7), or with the cytosolic mRNA and inhibiting its expression by a number of possible mechanisms: by forming a double-stranded RNA which is rapidly degraded (8); by hybridizing with the 5' untranslated sequence, including the ribosome binding site, and preventing the initiation of translation (9); or even by preventing post-transcriptional activation of dormant mRNAs (10). Thus, hybridization to the 5' untranslated region of a number of mRNAs is necessary for efficient inhibition.

We have previously used antisense RNA to inhibit the increase in β-glucuronidase activity observed between the four-cell and the blastocyst stages of mouse preimplantation development (5), and obtained approximately 75% inhibition. The antisense RNA employed in that study was synthesized on a cDNA that lacked a large portion of the 5' region, including the untranslated sequence. We have used the same experimental approach to test whether an antisense RNA complementary to the 5' region of β-glucuronidase yields a

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higher degree of inhibition. Since the complete characterization and sequencing of the gene for mouse β-glucuronidase had not been performed when we started our experiments, we were not then able to use a construct containing the 5' portion of the gene for the synthesis of antisense RNA molecules. Thus, we assumed that hybridization of the 5' region would give us the highest degree of inhibition and, using the antisense RNA technique as a test, searched for the 5' untranslated region of β-glucuronidase in a fragment of genomic DNA, cosBGus1, which had been partially characterized by Roger Ganschow. Our approach allowed us to isolate a 0.35 kb long Pst I fragment which corresponds to the fragment containing the 5' untranslated sequence, the mRNA cap site and approximately 180 bp of coding sequence of the mouse β-glucuronidase structural gene, as reported by Funkenstein et al. (11).

MATERIALS AND METHODS

Materials: Mice were purchased from the Jackson laboratory and bred in our colony; pregnant mare serum gonadotrophin (PMSG), human chorionic gonadotropin (hCG) and all chemicals were from Sigma; restriction enzymes were from Bethesda Research Laboratories or from Boehringer-Mannheim Biochemicals; riboprobe vector pGEM-4 and in vitro transcription kits were from Promega Biotec; [^{32}P]ATP and [^{32}P]UTP were from Amersham; modified Whitten's media M1 and M2 were prepared as previously described (5); embryo culture was performed at 37°C in an atmosphere of 5% O_2, 5% CO_2, 90% N_2.

Methods - Embryo Collection and Treatment: Twenty-one day old (C57Bl/6J x SJL/J) F1 female mice were superovulated as previously described (5) and mated with males of the same cross. The following midnight was taken as the time of coitus. One-cell embryos were collected from the oviducts of the plugged females at 12-15 hours post coitum (hpc) in medium M1 containing 0.5 mg/ml Hyaluronidase. They were transferred in M1 and subjected to injection after culture in medium M2 to the four-cell stage. After the injection, the embryos were cultured in M2 until 90 hpc and then assayed as single embryos for β-glucuronidase as previously described (5).

Construct Preparation: CosBGus1, a mouse β-glucuronidase genomic clone, contained in pJB8, was obtained from Dr. Miriam Meisler. The clone was analyzed by the partial digestion, end-fragment hybridization method of restriction enzyme mapping (12) with Eco RI, Bam HI, Hind III, and Pst I. Subsequently, it was completely digested with the same enzymes in single or double reactions in order to generate a variety of fragments for subclones. These were ligated to pGEM-4 in order to produce constructs that enabled us to synthesize both RNA strands. Some of the subclones were localized on the restriction map according to their size and restriction pattern, and then used for RNA synthesis: the constructs were linearized with the suitable enzymes (see table) and used with T7 or SP6 RNA polymerases as described my Melton et al. (13). Each RNA strand was separately used for embryo injection at the four-cell stage, as described previously (5).

Synthesis of β-glucuronidase oligodeoxynucleotide and search for the 5' region of the gene in the subclones: At the time of these experiments, the sequence of mouse β-glucuronidase was not yet known: by comparing the published sequences of the human (14) and rat (15) genes, we found a conserved 20 nucleotide stretch (5'-GCTGCAGGGCGGGATGCTGT-3') in the 5' region. An antisense oligodeoxynucleotide specific for this sequence was synthesized in order to search for the homologous region in the subcloned fragments of cosBGus1. For this purpose, five ug of cosBGus1 were separately digested with

Eco RI, Bam HI, Hind III, and Pst I. They were subsequently run on a 0.9% agarose gel, blotted on Gene Screen Plus nylon membrane, and hybridized to the labeled oligodeoxynucleotide. The filter was washed to a stringency of 0.5% SSC at 65°C for 30 min, and exposed for one day at -70°C with an intensifying screen. One ug of each construct was also run on a similar gel and treated as described above for hybridization with the oligodeoxynucleotide.

RESULTS AND DISCUSSION

We have applied the antisense RNA technique to the analysis of a cloned gene: mouse β-glucuronidase. Using a microfluorometric assay, Wudl and Chapman (16) and our group (5) showed that the activity of this lysosomal and microsomal enzyme undergoes a dramatic increase during the preimplantation phase of development of mouse embryos, both in vivo and in vitro. This system is suitable for antisense RNA inhibition for two reasons: a) the increase in activity is the result of the activation of transcription of the embryonic genome, starting at the four-cell stage (5); and b) the biological effect of the antisense manipulation can be measured on single embryos, so that changes in the experimental conditions can be readily analyzed. Our initial goal was to obtain a virtually complete inhibition of β-glucuronidase activity in order to study the role of this enzyme during mouse preimplantation development. We were previously able to reduce the activity of the enzyme but the highest degree of inhibition produced by the antisense RNA to the cDNA was 75%. The 5' region of mouse β-glucuronidase was not then available because the complete gene had not been successfully analyzed and sequenced.

We have now studied a genomic clone of mouse β-glucuronidase, named cosBGus1, in an attempt to isolate the 5' untranslated region of the gene. This cosmid was known to contain the whole coding sequence of β-glucuronidase in a 14 kb long region (17). First we determined a partial restriction map of the cosmid (Fig. 1). We then isolated subfragments of the cosmid by single or double digestions with the same restriction enzymes used for mapping followed by ligation to pGEM-4. This allowed us to start the antisense analysis with some of the subcloned fragments: the two RNA strands were routinely synthesized using the T7 or SP6 RNA polymerases and separately injected in the embryos at the four-cell stage. The embryos were cultured to the blastocyst stage and assayed for β-glucuronidase activity as previously described (Table 1). A clone (pB4) which we localized in the 3' end of cosBGus1 was used as a control. The initial results with 4 clones were not encouraging because

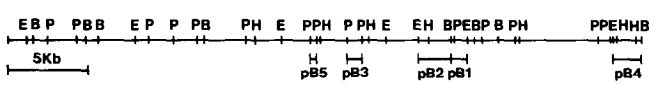

Fig. 1. Partial restriction map of pCosBGus1. E, Eco RI; B, Bam HI; P, Pst I; and H, Hind III. Subclones studies by the antisense approach are indicated.

the highest degree of inhibition found with a clone was 47% and there were many more clones to study. Therefore, we supplemented the approach by analyzing the subclones for the presence of a 20 nucleotide sequence, conserved in the human and rat gene, and localized near the beginning of the coding region. As we were not sure that the same sequence would be present in the mouse, we digested cosBGus1 with restriction enzymes and hybridized the separated fragments with the synthetic oligonucleotide. This analysis showed that a homologous sequence was found in a 7 kb Eco RI fragment, a 12 kb Bam HI fragment, and a 4.3 kb Hind III fragment. In the case of Pst I there was hybridization of more than one band, but this could be explained by a partial digestion of the cosmid by this enzyme. The labeled fragments had a size of 0.35, 0.7, 1.7, 2.2, 2.6, 3 and 4.3 kb. When compared to the restriction map, the results showed that the oligodeoxynucleotide recognized a region localized between nucleotides 16,000 and 19,000 of cosBGus1. We then searched our subclones for this homologous sequence by hybridization to the oligodeoxynucleotide as described under Methods. Using the same stringency conditions, we observed hybridization of the labeled oligonucleotide with only a subclone containing a 0.35 kb long Pst I fragment. This subclone (pB5) was subsequently analyzed by the antisense RNA approach and, as shown in Table 1, the degree of inhibition we observed with one of the two RNA strands was the highest we have found (89%). Recently, the complete sequence of mouse β-glucuronidase has been published (11,17). This data allowed us to determine that the oligodeoxynucleotide we used for our study is not perfectly conserved: the corresponding sequence in the mouse being: 5'-*TCTG*AAGGGCGGGATGCTGT-3' (asterisks indicate variant bp). The homology is 90% and the last fifteen nucleotides are all conserved. Furthermore, the

TABLE 1
β-Glucuronidase Activity in Single Blastocysts Devleoped In Vitro after RNA Injection at the Four-Cell Stage

RNA Injected	n	Activity	%
none-control	9	300±15.61	100.00
pB1B*	9	159±18.27	53.00
pB1E	9	280±17.32	93.33
pB2E	9	308±14.78	102.67
pB2B	8	273±24.31	91.00
pB3H	11	181±13.21	60.33
pB3B	8	302±25.64	100.67
pB4E	10	295±15.77	98.33
pB4B	12	281±18.69	93.67
pB5H	16	34± 5.28	11.33
pB5B	12	270±21.25	90.00

Activity: moles x 10^{-15}/hr/embryo

*The last letter indicates the enzyme used to linearize the subclone: B, Bam HI; E, Eco RI; H, Hind III.

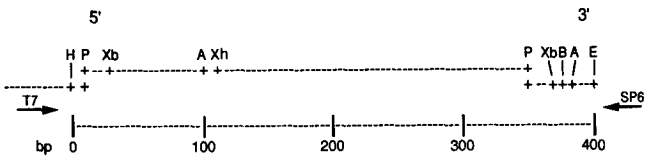

Fig. 2. Partial restriction map of pB5. T7 and SP6, direction of transcription by respective polymerases; Xb, Xba I; A, Ava I; Xh, Xho I; other abbreviations as in Fig. 1.

characterization of the gene by Funkenstein et al. (11) showed that the mRNA cap site is present in a 0.35 kb long Pst I fragment. Further characterization of our subclone by restriction analysis indicated that the two fragments of the gene correspond, and that the RNA strand providing the high degree of inhibition measured is the antisense one (Fig. 2).

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