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Functional significance of sequences following the TATA box of an immunoglobulin promoter studied by random mutagenesis

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We have investigated the importance of sequences downstream to the TATA box of an immunoglobulin promoter by transfection and in vitro transcription assays. A sequence from −11 to +10 with respect to the transcriptional start site was synthesised by a procedure allowing for random misincorporation of nucleotides. The pool of mutant oligonucleotides was cloned into the respective position of a vector carrying a fusion of a synthetic immunoglobulin heavy chain promoter with the human growth hormone gene. From 200 clones sequenced, 115 were mutants with at least one nucleotide exchange in every position. Whereas most mutations are of minor functional importance, changes at or near the transcriptional start site reduce the promoter activity considerably.

Promoter; Oligonucleotide synthesis; Mutant; Transcription

1. INTRODUCTION

Promoters of higher eucaryotes are very complex sequences consisting of individual regions responsible for the positioning of RNA polymerase in the initiation complex and for the binding of activating and repressing cellular transcription factors. The region between −40 and +10 with respect to the transcriptional start site, the so-called selector sequence, is the core of a mammalian promoter [1]. Within the selector sequence the TATA box is essential for the positioning of the RNA polymerase II, at least in vitro [1,2]. Nothing is known yet about the significance of sequences downstream from the TATA box.

The immunoglobulin heavy chain (IgH) promoter is known to be one of the simplest promoters with regard to its use for driving transient gene expression in B cells. In addition to the selector sequence it requires only the presence of the socalled octamer motiv [1,3]. For the optimization of gene expression, it is essential to know the sequence characteristics required for function of the IgH promoter. To this end we have constructed a synthetic IgH promoter based on published sequences [4] with short cassettes spanning the region between −80 and +10 connected by suitable restriction sites. It is efficient in driving expression of the human growth hormone gene in B cells. In this paper we report on the mutational analysis of the region between −11 and +10.

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2. EXPERIMENTAL

Plasmid vector and mutational DNA synthesis

The genomic human growth hormone gene fragment without promoter was cloned into the *Hind* II site of pUC19. The IgH promoter was constructed according to the published sequence [4] from two oligonucleotides overlapping by 8 nucleotides (ATGGTGGT) and was cloned between the *Eco* RI and *Xba* I sites of pUCGH in front of the hGH gene.

The two strands of the *Xba* I/*Sal* I fragment were synthesised according to the procedure of Hutchison et al. [5] using an Applied Biosystems DNA Synthesizer model 380 on the basis of methoxyamidites. To 2.9 ml of the main phosphoamidite solution (0.13 M) in each position 68 μl of the other three amidite solutions were added resulting in an impurity of 6.6% for each solution. For synthesizing the cohesive ends non-contaminated solutions were used. The oligonucleotides were purified by HPLC, annealed, phosphorylated and cloned between the *Sal* I and *Xba* I sites of the dephosphorylated vector pIgHGH. Individual plasmid minipreparations were sequenced by the chain termination method [6].

Transfection of B cells

Mouse myeloma cell line Sp2/0 Ag14 was grown in RPMI1640 medium/10% fetal calf serum/100 μg/ml of gentamycin. 500 000 cells were seeded in 10-cm Petri dishes 24 h before transfection. 7 μg of every mutant plasmid were mixed with 0.5 μg of pRSVcat [7] as an internal standard, precipitated with ethanol, resuspended in 250 μl of RPMI1640 + 25 mM HEPES (pH 7.15), added dropwise to 250 μl of RPMI/HEPES (pH 7.25) + 1 mg/ml DEAE-dextran (M_r 500 000; Pharmacia) with mixing and added to the cells for 30 min. After washing 7 ml medium with serum + 0.1 mM chloroquine were added for 3.5 h. Cells were washed and incubated with fresh medium for 36 h.

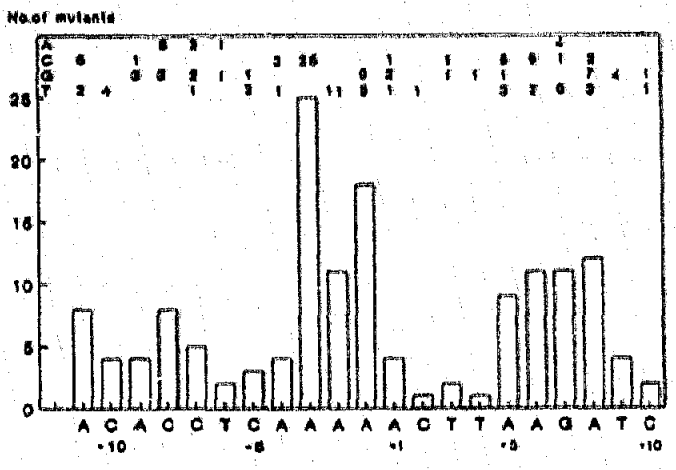

Fig. 1. Distribution of mutations within a 21-bp sequence of the IgH promoter synthesized by a mutational method [5]. A total of 149 mutations were identified in 115 mutant clones. The numbers of the respective nucleotide exchanges are given for each position on top of the diagram.

Expression assays

An ELISA was used where a monoclonal anti-hGH antibody was fixed to a microtiter plate. Specific binding of a rabbit anti-hGH antiserum was detected by the peroxidase technique. A commercial hGH (Serono) was used as a standard. Linear response was observed between 0.5 and 10 μg/ml.

Total RNA was extracted from transfected cells by the guanidinium-phenol method [8], denatured with glyoxal and immobilized on two nitrocellulose filters using a slot blot device (Schleicher and Schuell). Sense RNA transcribed in vitro was used as a standard. Antisense RNA probes were also generated by in vitro transcription using T7-RNA polymerase. Hybridization was done in the phosphate buffer system [9].

CAT enzyme activities were determined [10] in triplicate in extracts of cells which were assayed for hGH secretion. After thin layer chromatography and scintillation counting CAT activities were used to normalize the hGH values.

3. RESULTS

Analysis of mutants

Mutants within the 21-bp sequence of interest were generated by misincorporational synthesis of oligonucleotides and subsequent cloning. Out of 200 clones sequenced, 115 were found to be mutants. 97 clones had one nucleotide exchanged; the others 2 or 3 exchanges. The distribution of mutations is depicted in Fig. 1. At least one mutation has been found in each of the 21 positions. In 10 positions only one of the three possible exchanges was found and only 42 of the possible 63 single nucleotide exchanges were detected. Fig. 1 clearly shows that the distribution of mutations is not random. Most striking is the prevalence of the A to C transversion at position −3 within a tract of 5 × A. As this is not the result of a non-random synthesis (data not shown) it seems to reflect a selective advantage of the sequence ACAAA over AAAAA within the plasmid sequence during transformation and clone selection.

Cellular expression assay

We have constructed an expression vector pIgHGH which contains a synthetic 90 bp IgH-promoter fragment in front of the human growth hormone gene (Fig. 2). Individual cassettes of the promoter can be replaced by suitable sequences. The vector allows for efficient transient expression and secretion of hGH in B cells (200 ng/ml/day). The incorporation of restriction sites in the original sequence [4] does not affect the level of expression (data not shown).

The *Xba* I/*Sal* I fragment of pIgHGH was replaced by the mixture of mutant fragments described above. From the 115 mutants identified we have chosen 20 clones for functional testing. B cells were transfected and the concentration of hGH in the culture medium was determined by ELISA 36 h after transfection (Fig. 3). For most mutants the expression levels are within the range of 65–90% of the wild-type promoter sequence. However, mutations in position −1 reduce the transcriptional activity to 15–20% and the A to C transversion in position −3 results in a slight increase over wild-type activity (120%). A reduction of hGH synthesis under 50% was observed for mutations in positions +8 and +9.

The levels of hGH specific RNA correlate with the protein levels in mutants with nucleotide exchanges

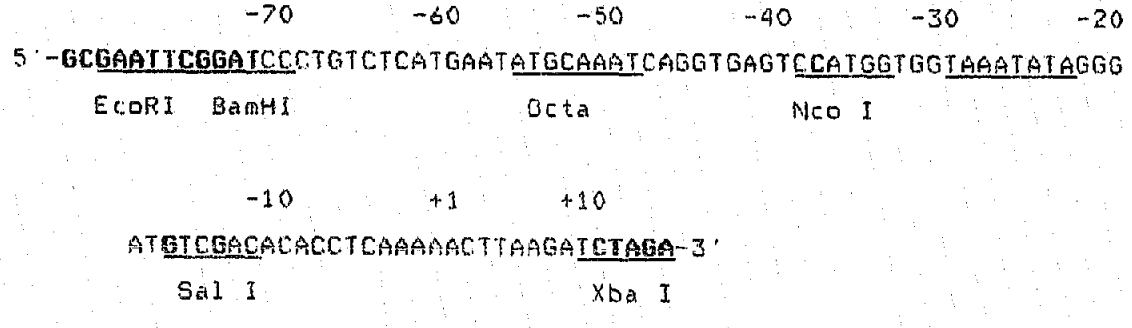

Fig. 2. Sequence of the synthetic IgH promoter. The positions of restriction sites which differ from the original sequence [4] are marked by bold letters.

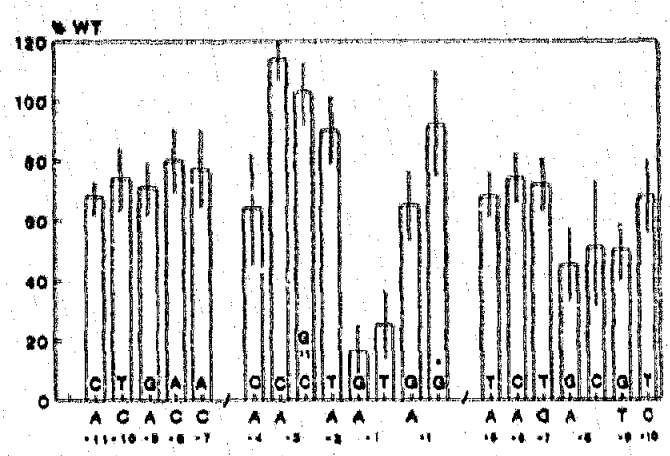

Fig. 3. Relative activities of mutant IgH promoters as measured by secretion of hGH from B cells 36 h after transfection. The 100% value of the wild-type promoter corresponds to 200 ng/ml, 150 ng/ml and 120 ng/ml, respectively, in 3 independent experiments. All numbers are mean values of 3 experiments with 3 dishes for each DNA sample.

upstream of the transcriptional start site. The ratio of RNA to protein is higher in clones with mutations downstream of the start site (Fig. 4) suggesting a decrease in the translational efficiency.

4. DISCUSSION

We have used the method of Hutchison et al. [5] to generate random mutations within a region downstream of the IgH promoter supporting the usefulness of this method. However, the abundant occurrence of one particular mutation, the A to C transversion in position −3, suggests that biological selection mechanisms have to be taken into account. The overrepresentation of this mutation (25 of 149 mutations in 200 clones analysed) is likely to be due to a higher stability of the plasmid in the selection procedure. This mutation also results in a canonical CA box [11] which is likely to function as a transcriptional initiator. This could be the reason why this mutation actually resulted in an increase of the promoter activity over the wild-type level (120%). The distance of this CA dinucleotide from the TATA box is within the usual limits known from other promoters [1].

The most pronounced effect of all mutations was in position −1. Two different nucleotide exchanges in this position resulted in the same reduction of promoter activity detectable on the protein (Fig. 3) as well as on the RNA level (Fig. 4). It seems very likely that formation of the transcription initiation complex is disturbed by this mutation as suggested by gel retardation experiments (unpublished observations). No significant effects on promoter activity could be found in the region between −11 and −3. Some mutations downstream of the transcriptional start site seem to have a positive effect on transcription (Fig. 4) and a negative one on translation (compare Figs. 3 and 4). The effect of mutations in this region will be further studied by linking the sequence to other test genes.

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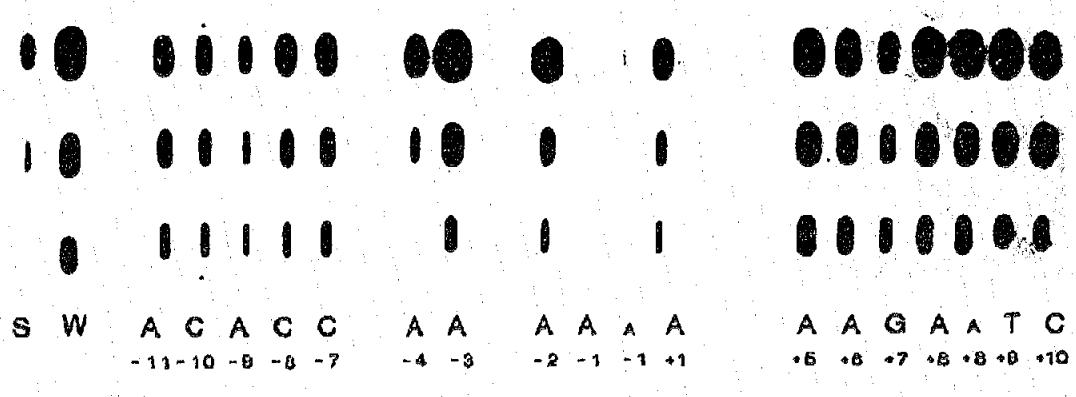

Fig. 4. Slot blot hybridization of hGH-specific RNA extracted from B cells transfected and analysed for hGH protein production as in Fig. 3. (w = wild-type promoter; s = standard sense RNA, 30, 10 and 5 pg)