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Efficient generation of functional transgenes by homologous recombination in murine zygotes

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ABSTRACT

To assess the feasibility of generating functional transgenes directly via homologous recombination between microinjected DNA fragments, three overlapping genomic DNA fragments, together constituting the human serum albumin (hSA) gene, were coinjected into murine zygotes. The resulting transgenic mice were analyzed for structure and expression of the transgene. All transgenic mice carried recombined hSA DNA fragments and 74% contained a reconstituted hSA gene. HSA expression could be detected in liver and serum in most (72%) of these animals. Only correctly sized hSA transcripts were observed. Transgenic hSA could not be distinguished from the human serum-derived protein by radioimmunoassay or Western blotting. The high frequency and accuracy of homologous recombination in murine zygotes reported here allows the efficient generation of relatively large transgenes.

INTRODUCTION

Many eukaryotic genes span large regions of DNA and are controlled by regulatory sequences located at relatively large distances from the structural gene. The size of such genes has seriously hampered their analysis and manipulation. Generation and microinjection of gene constructs for the production of transgenic animals has been limited to transgenes having a size no greater than 45 kb. However, to achieve high level, correctly regulated expression of transgenes it is often desirable to express genomic sequences rather than much smaller cDNA constructs (1, 2).

Recent developments in cloning techniques allow the experimental manipulation of DNA fragments of 100 kb (using bacteriophage P1 cloning vectors or F-factorbased plasmids; 3, 4) up to 1000 kb (in yeast artificial chromosomes; 5). However, the generation of gene constructs via these methods is complicated and microinjection of large DNA fragments is troublesome due to mechanical shearing of the DNA.

In cultured eukaryotic cells, reconstitution of selectable genes by extrachromosomal homologous recombination (ECR) between two overlapping cotransferred subclones has been demonstrated (reviewed in 6). To assess the potential of ECR for the generation of large transgenes, we examined the occurence of this process in murine zygotes. Overlapping genomic DNA fragments were coinjected into murine zygotes and the frequency and accuracy of homologous recombination of these fragments was determined.

Our data demonstrate the feasibility of generating functional transgenes by this approach.

MATERIALS AND METHODS

Isolation of DNA fragments and generation of transgenic mice

Fragment I is a 17 kb partial EcoRI fragment isolated from λHAL-HA1 (14), containing 13 kb of 5′ flanking sequences and 3904 bp downstream of the transcription initiation site (+1). Fragment II is a 13.1 kb AhaII-SacI fragment isolated from λHAL-3W (15) and runs from +1405 to +14546 bp. Fragment III is a 6.7 kb XhoI-SacI fragment isolated from λHAL-H14 (14) running from +12694 bp to +19.4 kb and contains 2.5 kb of 3′ flanking sequence. The overlapping regions are 2.5 kb (fragment I and II) and 1.85 kb (fragment II and III). Both regions contain exons and introns. Fragments I, II and III span a 33 kb region comprising the 17.0 kb structural hSA gene. All 3 fragments were subcloned. Prior to microinjection, plasmid sequences were completely removed using the indicated restriction enzymes. The fragments were purified from low melting point agarose. In most cases (see table 1) fragments were blunt-ended with Klenow polymerase and subsequently dephosphorylated with bacterial alkaline phosphatase using standard procedures (16) to inhibit random end to end ligation. After this treatment the fragments were no longer ligation competent as determined by gel electrophoresis of test ligations. Microinjection was carried out as described previously (17). The fragments were mixed in equimolar amounts and microinjected. The total amount of microinjected DNA was similar to the amount used for microinjection of single fragments (17).

Southern blot analysis

Chromosomal DNA was isolated either from various parts (including liver) of 3 day old mice, or from tails of adult mice. Transgenic animals were identified (27 in total) by Southern blotting using a full length hSA cDNA probe. Chromosomal DNA (10 μg) was digested with NcoI and BstEII (fig 2), NcoI + HindIII (double digest; results not shown) and HincII (results not shown). Digested DNA was run on a 0.6% agarose gel, transferred to Highbond-N (Amersham) and hybridized to a full length hSA cDNA probe labeled by random priming.

RT-PCR analysis

A reverse transcriptase (RT) reaction was performed on 1 μg of total liver RNA as described (18). PCR was performed for 35 cycles of denaturation (1', 92°C), annealing (1', 41°C) and extending (2', 72°C) essentially as described (18). The $MgCl_2$ concentration was 2.5 mM. Sequence of forward primer (from 13853 to 13878 in exon 12, ref. 13): 5'-**CAGAGTCACC-AAATGCTGCACAGA**-3'; Reverse primer (from 15964 to 15991 in exon 14, ref. 13): 5'-**AGCTTGACTTGCAGCAA-CAAGTTTTTT**-3'. Boldface indicates differences between hSA and mSA sequence. These primers were selected for their low homology to mSA sequences.

Samples were separated on a 6% polyacrylamide gel.

The reportedly polymorphic SacI site in exon 13 of the hSA gene (7) is not present in the hSA cDNA clone used as a hybridization probe in this study. Since the amplified band did contain this SacI site it could not have been derived from contaminating hSA cDNA. PCR directly on RNA (or RT-PCR without addition of reverse transcriptase, etc.) did not result in amplification.

Northern blot analysis

Total and polyA$^+$ RNA fractions were isolated from mouse livers as described (16); run on a 1% formamide gel; transferred to Highbond-N and hybridized to an end-labeled hSA specific synthetic primer complementary to hSA exon 14 (also used in RT-PCR, see above) or to a full length hSA cDNA probe. Hybridization was performed in a mix containing 6×SSPE (16), 2×Dernhardt's solution (16), 25 mM $KNaPO_4$, pH 7.4, 100 μg/ml yeast tRNA, 0.1% SDS and 0.1 mM ATP for 16 hrs at 37°C. Prehybridization was performed in the same mix for 30 min. at 37°C.

Radioimmunoassay (RIA)

RIA procedures were essentially as described (19). A suspension of monoclonal anti-hSA antibodies (CL 2513A; Cedarlane Laboratories; Ontario, Canada) coupled to Sepharose (Pharmacia) was incubated with mouse serum. Sepharose-bound hSA was quantified by incubation with affinity purified anti-hSA polyclonal rabbit antibodies (Sigma) labeled with ^{125}I. Prior to

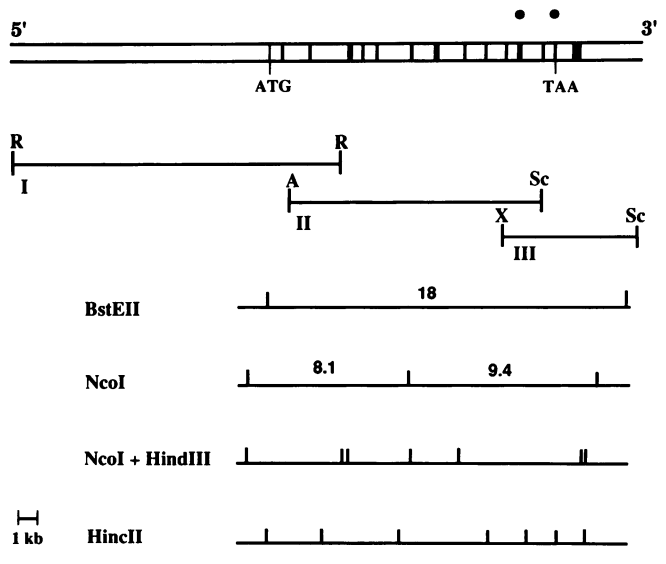

Figure 1. Schematic representation of the human serum albumin gene, the DNA fragments (I, II and III) coinjected into murine zygotes and restriction maps of part of the hSA gene. Black boxes indicate exons, white regions represent introns and flanking sequences. Black dots indicate position of primers used in RT-PCR (fig 3a). Restriction sites used to generate unique fragments diagnostic of homologous recombination events are indicated in the lower part of the figure. ATG: translation initiation codon; TAA: translation termination codon; R: EcoRI; A: AhaII; X: XhoI; Sc: SacI.

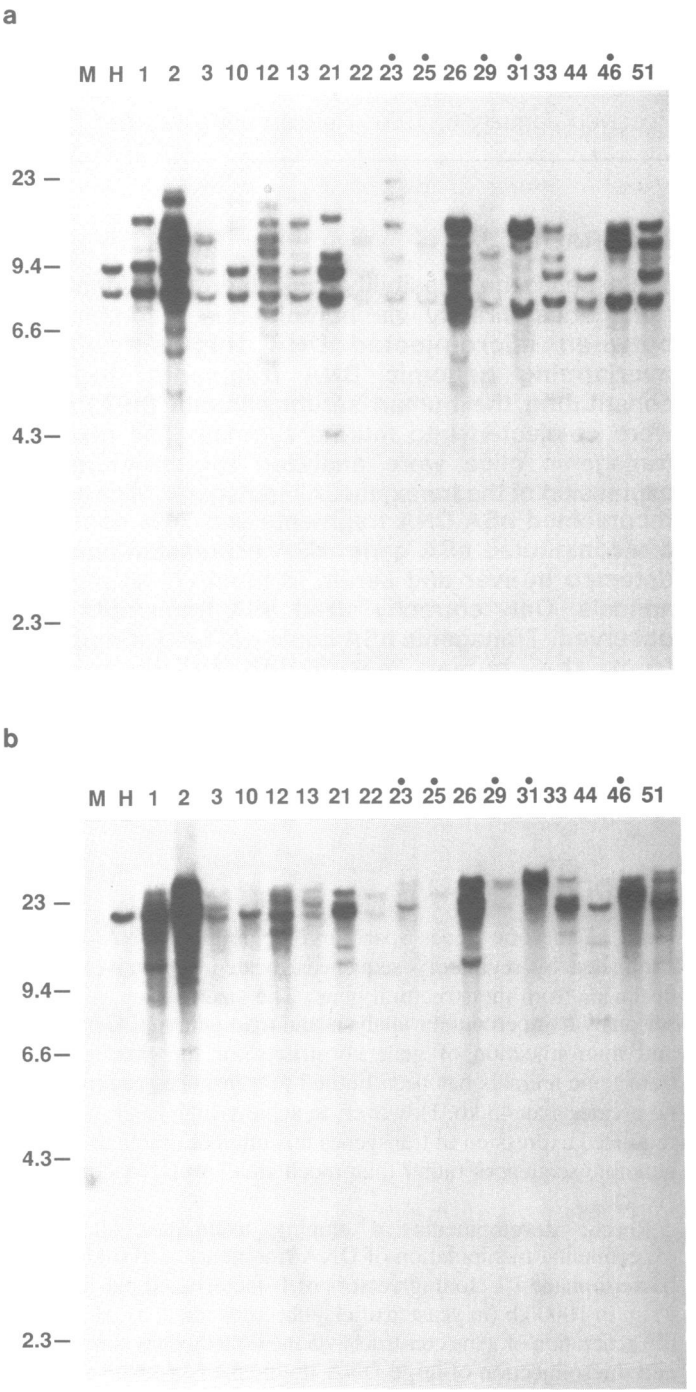

Figure 2. Southern blot analysis of chromosomal DNA from mice transgenic for hSA gene fragments. A full lenght hSA cDNA was used as a probe. Results are shown for 17 mice. a: NcoI digest; fig 2b: BstEII digest. M: control mouse DNA (10 μg); H: human DNA (10 μg). Numbers: DNA from transgenic mice; dots indicate DNA from mice not containing the intact hSA gene.

radioiodination, these purified antibodies were incubated with NMS-Sepharose to remove mSA-cross-reactive antibodies. Subsequently, Sepharose-bound radioactivity was measured. Results are expressed as percentage of the labeled antibodies added.

The levels of hSA in the sera of transgenic mice were calculated from the parallel dose-response curves. The lower limit of detection of this RIA was ca. 2 ng.

Western blotting

Procedures were essentially as described previously (20). HSA was immunoprecipitated with Sepharose-bound monoclonal anti-hSA antibodies and dissociated into 10 µl of non-reducing SDS-sample buffer. One µl samples were analyzed by SDS-PAGE (10−15% w/v) followed by immunoblotting with polyclonal 125I-anti hSA antibodies and autoradiography.

RESULTS

Reconstitution of the hSA gene

As a model system for our studies the human serum albumin (hSA) gene was used (fig 1). Three overlapping DNA fragments were coinjected into fertilized murine oocytes. These fragments together comprise the structural genomic hSA gene with 13.0 kb of 5′ and 2.5 kb of 3′ flanking sequences. The overlapping regions are 2.5 kb (fragments I and II) and 1.85 kb (fragments II and III). Since none of the individual fragments contains the entire coding region of the hSA gene, homologous recombination of all 3 fragments is required to reconstitute a functional hSA gene.

After several rounds of microinjection, 107 mice were born and analyzed by Southern blotting for hSA transgene integration. DNA from each of the resulting 27 transgenic mice was analyzed to determine the structure of the transgene locus.

Hybridizing bands of 9.4 and 8.1 kb (NcoI digest, fig 2a) or 18 kb (BstEII digest, fig 2b) are present only when the coinjected DNA fragments have recombined properly (fig 1). The 8.1 and 9.4 kb fragments contain the overlapping regions from fragments I and II, and from II and III, respectively. The 18 kb BstEII fragment spans both overlapping regions and contains the complete 17 kb structural hSA gene. Its presence reveales that the complete hSA gene has been reconstituted (fig 2b).

A total of 20 transgenic mice appeared to contain the intact hSA gene locus as judged by the presence of all diagnostic bands (fig 2; table I). Of the remaining 7 mice, 6 had incorporated DNA resulting from recombination between fragments I and II (23, 25, 29, 31 and 46 in fig 2), one contained the recombinant product of II and III (not shown). At least 4 mice with the intact locus (22, 53, 63, and 67) were mosaic as judged by the absence of hSA DNA from some tissues or by copy numbers of less than one (data not shown).

The predominance of the 8.1 kb band indicates that fragments I and II have recombined more frequently than fragments II and

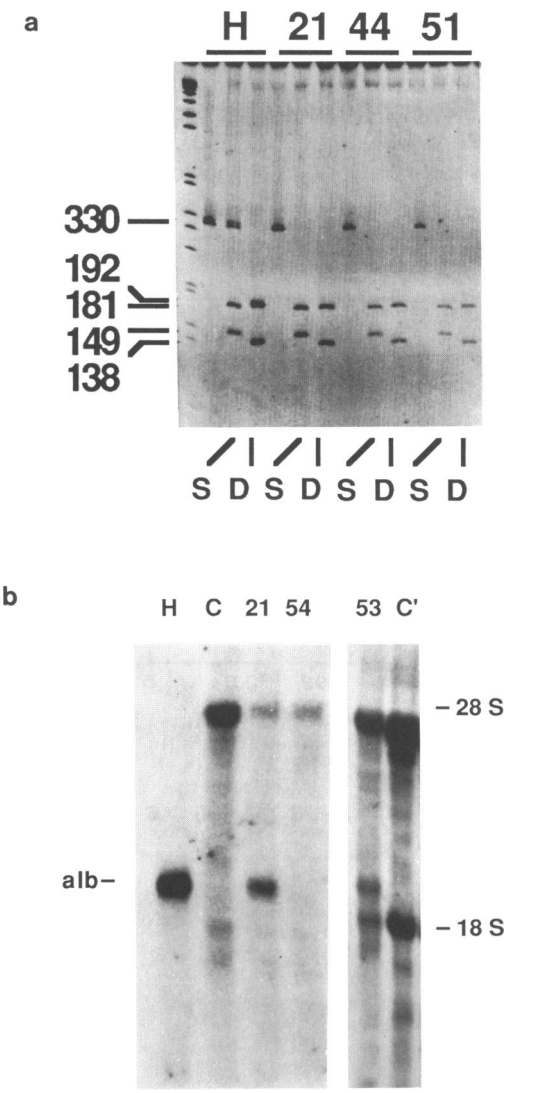

Figure 3. RT-PCR and Northern blot analysis of liver RNA from hSA transgenic mice. (a) RT-PCR analysis on liver RNA. Lanes H: DNA amplified from human liver RNA. Lanes 21, 44 and 51: DNA amplified from liver RNA of hSA transgenic mice. S: amplified DNA digested with SacI; D: amplified DNA cut with DdeI. Size marker: 1 kb BRL ladder. (b) Northern blot analysis of liver RNA. Lane H: total human liver RNA (1 µg); Lane C: control mouse, polyA$^+$ selected liver RNA (20 µg); lane 21: mouse transgenic for the intact hSA gene (10 µg polyA$^+$ liver RNA); lane 54: mouse transgenic for recombined hSA DNA fragments I and II (10 µg polyA$^+$ RNA); Lane 53: mouse transgenic for the hSA gene (20 µg total RNA); lane C′: control mouse RNA (20 µg total RNA). An end-labeled hSA specific primer complementary to part of hSA exon 14 was used as a probe. To increase sensitivity and allow for detection of aberrant transcripts lacking exon 14, similar blots were also hybridized to a full length hSA cDNA probe. This probe cross-hybridized strongly to the mSA mRNA. Aberrant hSA transcripts were not observed (not shown). Alb indicates position of hSA mRNA. 18 S and 28 S ribosomal RNA bands are visible due to aspecific hybridization of the labeled primer. Results are representative for all mice. Mouse 21 and 53 displayed relatively high expression levels.

Table 1. Frequencies of homologous recombination and hSA expression

	treated* fragments		untreated fragments		total	
	#	%	#	%	#	%
Transgenic mice	23/73	32	4/34	12	27/107	25
Mice containing intact hSA locus	16/23	70	4/4	100	20/27	74
Mice expressing hSA	9/14**	64	4/4	100	13/18	72

#: number of positive mice per number of mice assayed

*: Prior to microinjection, DNA fragments were subjected to treatment with Klenow-polymerase and bacterial alkaline phosphatase to generate dephosphorylated blunt ends.

**: At least 3 out of 5 nonexpressing mice were mosaic for the intact hSA gene (see legend fig 2). Nonexpressing mice for which analysis is shown in fig 2 are numbers 22 (mosaic), 26 and 33.

III. Similar analyses using different restriction enzymes (fig 1; see materials and methods section) confirmed these findings. Clearly, every transgenic mouse contained recombined DNA fragments.

Although all DNA samples displayed several additional hybridizing bands of varying size and intensity, the majority of the integrated DNA molecules resulted from homologous recombination between overlapping fragments (fig 2). Head-to-tail concatamers of individual fragments were not observed (data not shown). Apart from the bands characteristic of the hSA locus, hybridizing bands reappearing in most mice were not observed.

Coinjection of blunt-ended, dephosphorylated DNA fragments instead of untreated fragments did not enhance the process of homologous recombination (table 1), nor that of head-to-tail concatamer formation (data not shown).

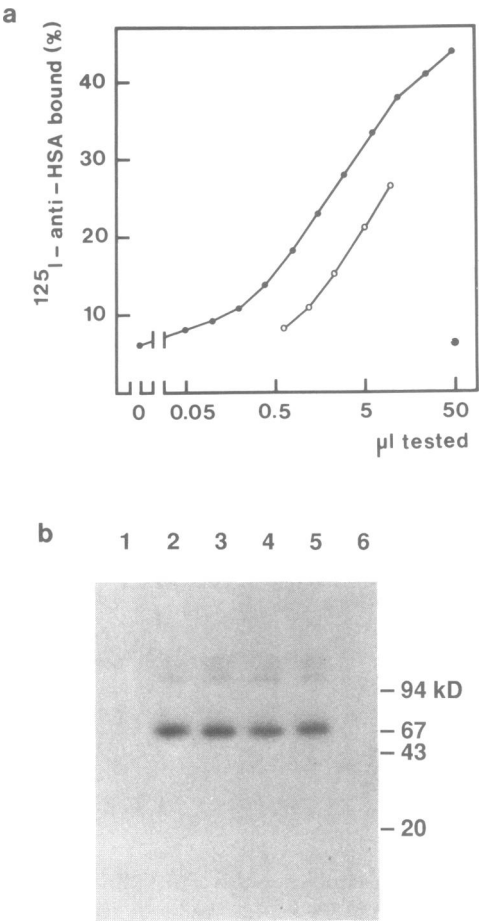

Figure 4. Characterization of transgenic hSA by RIA and Western blotting. (a) Radioimmunoassay for hSA. Sepharose coupled anti-hSA antibodies were incubated with normal mouse serum (NMS) to which purified hSA (Sigma) had been added to 10 µg/ml (black dots); with transgenic mouse serum (representative curve of mouse 53 serum; open circles) or with NMS only (large black dot). The amount of serum tested is indicated (abscissa). Results are expressed as percentage of the labeled antibodies added. HSA was only detected in serum from mice containing the intact hSA gene. (b) SDS-PAGE and immunoblotting analysis of transgenic hSA. Immunoprecipitates from 100 µl samples of: NMS (lane 1), mouse 53 serum (containing 2.5 µg/ml hSA; lane 2), NMS to which 250 ng of purified hSA had been added (lane 3), purified hSA (250 ng, lane 4) and purified mSA (250 ng, lane 6). Lane 5: 25 ng of purified hSA directly subjected to SDS-PAGE.

HSA expression and accuracy of recombination

To investigate whether the homologous recombination process had led to the reconstitution of a functional hSA gene, hSA expression was analyzed at the transcriptional level. Polymerase chain reactions (PCR) were performed following a reverse transcriptase (RT) reaction on 16 liver RNA samples from transgenic mice, 6 of which contained recombined hSA fragments but not the intact hSA locus. The primers used in PCR were complementary to part of hSA exons 12 and 14. The amplified region includes most of the transcribed sequences in the overlap between genomic fragments II and III (fig 1). Amplification of a 330 bp band (of which the 198 bp at the 5′ end are located in the overlap between fragments II and III) indicates correct homologous recombination between hSA gene fragments II and III. Digestion with SacI should yield bands of 181 and 149 bp, digestion with DdeI should result in bands of 192 and 138 bp (7; fig 3a).

A band of the correct size was amplified from RNA samples of 7 out of the 10 mice diagnosed to contain an intact hSA locus by Southern blotting. RT-PCR on control mouse liver RNA never resulted in amplification (not shown). Restriction analysis using SacI and DdeI confirmed the identity of the amplified band (fig 3a).

Analysis of total and polyA$^+$ selected liver RNA from each transgenic mouse by Northern blotting using a hSA specific probe confirmed these findings and demonstrated that the hSA transcripts were of the correct size (fig 3b). Aberrant transcripts were never observed. HSA transcripts were only detected in mice containing the intact hSA gene, at levels 10^4 to 10^5 lower than in human liver (data not shown).

To assess the accuracy of the homologous recombination event in more detail, a quantitative radioimmunoassay (RIA) for specific detection of hSA in mouse serum was developed (fig 4a). Serum samples from 24 out of the total 27 transgenic mice were analyzed. Of the 18 mice transgenic for the complete hSA gene, 13 expressed hSA (table 1). There was a complete correlation between RIA and RT-PCR results.

Expression levels ranged from 0.1 to 5.0 µg/ml, which is 10^4 to 5.10^5-fold lower than endogenous mouse serum albumin (mSA) and hSA levels in human serum (8). There was no correlation between the copy number of the intact hSA transgene and hSA expression levels (data not shown).

The parallel dose-response curves of hSA from transgenic mouse- and human serum reveal that the accessibility of antigenic determinants for the antibodies against hSA did not differ for transgenic- and normal hSA. This indicates that transgenic hSA is structurally equivalent to the human serum-derived protein (fig 4a).

SDS-PAGE and Western blot analysis of hSA from transgenic mouse serum revealed only correctly sized hSA (fig 4b).

DISCUSSION

In this study we demonstrate that homologous recombination between coinjected DNA fragments occurs with high frequency in murine zygotes and can be used to generate functional transgenes.

The head-to-tail tandem repeat integration pattern that is commonly observed when copies of a single DNA fragment are microinjected into cultured cells (9) or murine zygotes (10) probably stems from homologous recombination. In this study, head-to-tail concatamers of individual hSA gene fragments were not detected. Instead, homologous recombination between the three overlapping DNA molecules predominated and was detected

in 74% of the transgenic mice. Moreover, all transgenic mice contained hSA fragments resulting from homologous recombination between 2 overlapping fragments and the majority of the integrated DNA molecules were products of homologous recombination.

In most cases, recombination of fragments I and II predominated over recombination between fragments II and III, even when the hSA gene had been reconstituted. Since DNA fragments microinjected into murine zygotes usually integrate at the same chromosomal site, both completely- and partially reconstituted transgenes were probably present at the integration sites.

The complexity of the transgenic loci varied widely between transgenic mice. Apart from bands representing recombined hSA fragments, hybridizing bands of varying size and intensity were detected in most cases. Some bands must represent the junction between hSA- and chromosomal DNA. Other fragments could be a byproduct of the homologous recombination process. However, we consider this unlikely since such additional bands are not observed after injection of a single fragment, in which case homologous recombination between these identical DNA molecules will take place. The additional bands could represent combinations of the injected fragments in different orientations and different numbers of the individual fragments.

Theoretically, the 5 Alu repeats present in the hSA gene (7) might have been involved in homologous recombination, thereby giving rise to additional fragments. However, such fragments were not detected in mice transgenic for genomic hSA-based constructs injected as a single fragment and containing these repeats. The presence of Alu repeats apparently did not inhibit reconstitution of the hSA gene.

The frequency of extrachromosomal homologous recombination (ECR) between cotransferred plasmids in cultured mammalian cells varies widely (6). It has been suggested that ECR is coupled to the subsequent integration of the recombinant molecule into the cellular genome since ECR frequencies observed upon stable transfection are higher than observed in assays based on transient transfection (11). Therefore the percentage of mice containing recombined DNA fragments reported in this study does not necessarily reflect the frequency of ECR in murine zygotes.

The detection of correctly processed hSA transcripts and protein in the majority of the transgenic mice (table 1) proves that functional copies of the hSA gene have been reconstituted in these animals and indicates that the homologous recombination process is accurate.

HSA expression was not detected in every mouse carrying an intact hSA transgene. Theoretically, this could be caused by undetected rearrangements resulting from the homologous recombination process. However, a percentage of nonexpressing transgenic mice is commonly observed for transgenes. In addition, at least three out of five nonexpressing mice were mosaic for the hSA transgene in which case the transgene may not have been present in the liver.

The percentage of mosaic mice was not higher than observed for other transgenes in our lab (data not shown).

Our results indicate that regulatory elements directing hSA expression are present in the 33 kb hSA gene locus. To our knowledge, this is the first report describing expression of hSA in transgenic mice. Transgenic hSA could not be distinguished from the human serum-derived protein by radioimmunoassay or Western blotting.

The low hSA expression levels suggest that control elements necessary for high level expression are not present in the injected clones. It should be noted that the promoter region of the mSA gene only drives high level expression to the liver of transgenic mice when a far upstream enhancer is included (12, 13).

We have recently observed frequencies of homologous recombination similar to those described in this paper in two independent studies (unpublished results). A 56 kb transgene comprising a 40 kb human gene fused to a 16 kb bovine promoter region was generated by coinjection of two genomic DNA fragments overlapping by 4kb in the coding region. Neither of the individual fragments contained the entire coding sequence, and homologous recombination was required to reconstitute a functional gene. In 3 out of the 4 mice born homologous recombination had occurred, resulting in expression of a functional, human protein at high levels which had not been obtained using smaller transgenes.

In another experiment, a 30 kb bovine genomic DNA construct was extended at the 5′ end by coinjecting it with a 15 kb DNA fragment overlapping the other fragment by 2 kb. Homologous recombination was observed in 5 out of 6 mice.

The high frequency of homologous recombination in murine zygotes reported here greatly simplifies the generation of large transgenes based on genomic sequences rather than cDNA sequences. Using the coinjection procedure, the inclusion of large streches of 5′ or 3′ flanking sequences in transgenes no longer poses a problem. This will facilitate the identification of regulatory sequences and contribute to achieving high level, correctly regulated expression of transgenes. Although exact homology requirements and the effects of injecting more and larger overlapping DNA fragments remain to be determined, the coinjection method described here will be instrumental in producing transgenic animals having genotypes which were previously unattainable.

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