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Expression of genomic and cDNA transgenes after co-integration in transgenic mice

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In general, genomic transgenes are expressed efficiently in mice, while their cDNA-based transgenes are frequently silent. Clark *et al.* (1992) have shown that silent cDNA transgenes under the control of the sheep β-lactoglobulin promoter can be activated after co-injecting them with a genomic sheep β-lactoglobulin transgene. We have tested the general utility of this concept using mouse whey acidic protein (WAP) transgenes. Here we show that WAP cDNA transgenes are virtually silent in transgenic mice. In contrast, WAP transgenes containing all introns are expressed in approximately 50% of the lines at levels ranging from 1% to more than 100% of the endogenous RNA (McKnight *et al.*, 1992). When a WAP-genomic transgene was co-injected with a WAP-cDNA, basal activation of the cDNA was possible. However, the activity of the WAP-cDNA transgene did not exceed 1% of the WAP-genomic transgene. This suggests that a permissive integration site capable of supporting basal level transcription can be established, but further events are required for full activation of the transgene.

Introduction

Transgenic animals are useful tools for the study of genes and their functions (Jaenisch, 1988). Researchers interested in using transgenic animals often have only a cDNA at their disposal, or work with genes too large for practical manipulation. However, transgenes without intronic sequences are expressed poorly, if at all, whereas genomic transgenes are, in general, expressed efficiently (Palmiter and Brinster, 1986). Attempts have been made to enhance the expression of cDNA-based transgenes through the inclusion of homologous (Brinster *et al.* 1988; Whitelaw *et al.*, 1991) and heterologous (Choi *et al.* 1991; Palmiter *et al.*, 1991) introns. While the presence of some introns enhanced transgene expression, others had no effect, suggesting that splicing *per se* was not sufficient for efficient transgene expression.

The poor performance of cDNA derived transgenes could, in part, be caused by increased sensitivity to silencing by neighbouring genomic sequences. Clark and coworkers (Clark *et al.*, 1992) have reasoned that increased expression of cDNA-derived transgenes may be achieved through the manipulation of the integration site. They have shown in transgenic mice that an actively expressed sheep β-lactoglobulin gene created an 'environment' in which juxtaposed cDNA transgenes were expressed. However, the α1-antitrypsin and Factor IX cDNA transgenes appeared to respond differently, suggesting that the degree of activation may depend on the cDNA transgene used. To test further whether the concept of activating cDNA transgenes through their physical proximity to intron-containing genes is of general utility, we investigated the effect of a mouse WAP-genomic transgene on the expression of a juxtaposed WAP-cDNA transgene.

Materials and methods

Generation of transgenes

Three different WAP-based transgenes were constructed (Fig. 1). The first was a 7 kb genomic clone containing a

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2.4 kb promoter, four exons, three introns and 1.6 kb of 3′ flanking sequence. In order to distinguish this transgene from the endogenous WAP gene, a *Hin* dIII linker was cloned into the *Kpn* I site located at +24 in the gene (Burdon *et al.*, 1991). The second WAP transgene (WAP-delta 3) was made by deleting intron 3. The *Sal* I–*Bam* HI fragment from the genomic clone, which spans sequences from the *Sal* I site within the third exon and the *Bam* HI site within the fourth exon, had been replaced with a *Sal* I–*Bam* HI fragment from the cloned cDNA, yielding a final length of 6 kb. An *Eco* RV linker was inserted in the *Kpn* I site to identify the transgene. The third WAP transgene had all introns deleted resulting in a fragment 4.5 kb in length. It was generated by restricting the WAP-genomic clone with *Kpn* I and *Bam* HI, which cut within exon 1 and exon 4, respectively, and replacing this fragment with a *Kpn* I–*Bam* HI cDNA fragment. This cDNA transgene was tagged with a *Not* I linker in the *Kpn* I site. The introduction of *Hin* dIII, *Eco* RV and *Not* I linker sequences into the blunted *Kpn* I site does not interfere with the expression of WAP transgenes (unpublished results; Burdon *et al.*, 1991).

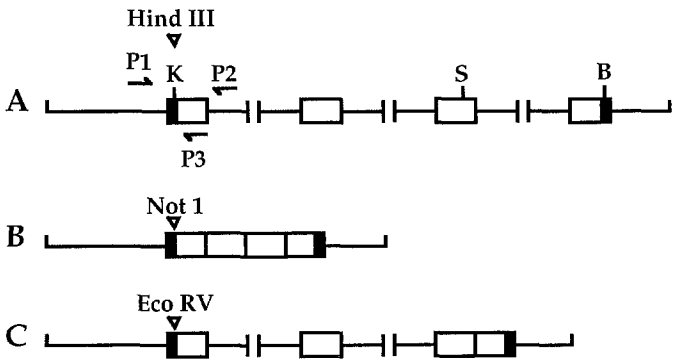

Fig. 1. Diagram of WAP constructs injected into fertilized mouse eggs. Panel A, the 7.2 kb WAP-genomic transgene and relevant restriction sites, K, *Kpn* I; S, *Sal* I; B, *Bam* HI; a *Hin* dIII linker was cloned into the *Kpn* I site; oligos P1, P2 and P3 were used to amplify exon 1 for PCR analysis. Panels B and C, WAP-cDNA and WAP-delta 3 constructs with *Not* I and *Eco* RV linkers inserted into the *Kpn* I site respectively.

Generation of transgenic mice

The transgene constructs were restricted from the vector by *Eco* RI digestion, separated on a 0.8% agarose TBE gel, and isolated from the gel using the Quiagen Qiax DNA purification kit. The purified fragments were then phenol extracted 1×, chloroform extracted 1×, ethanol precipitated, resuspended in 0.1 × TE pH 7.5 and spun through a Millipore Ultrafree-M 0.22 μm filter unit.

A solution containing approximately 5 μg per ml of the linear WAP-cDNA transgene was injected into 1031 pronuclear stage zygotes harvested from superovulated C57Bl/6 × SJL F_1 females. Then, 753 microinjected zygotes were transferred into 28 CB6 oestrous stage females that had been mated with vasectomized males the day before.

For the co-injection, the WAP-genomic, the WAP-delta 3 and the WAP-cDNA transgenes were mixed in equimolar amounts and microinjected into the male pronuclei of 1185 zygotes. Of these, 881 zygotes survived the injection process and were transferred into 35 oestrous stage recipient females. Fifteen litters were produced, resulting in the live birth of 121 pups.

Analysis of transgenic mice

DNA was prepared from mouse tails and screened by PCR for the presence of transgenes. Primers P1 (5′ TAGAGCTGTGCCAGCCTCTTC 3′) and P2 (5′ GTTCTCCAAGCCACACCCGG 3′) were used to amplify a 250 bp fragment spanning the first exon of the endogenous WAP gene and the WAP-genomic and WAP-delta 3 transgenes (Fig. 1). Primers P1 and P3 (5′ CTGAGTTGAAGACTTGTTCCTC 3′) were used to amplify the first exon of the cDNA transgene. Aliquots were restricted either with *Kpn* I (to identify the endogenous WAP gene), with *Eco* RV (for WAP-delta 3), with *Hin* dIII (for the WAP-genomic) or with *Not* I (for the WAP-cDNA), and the fragments were separated on an agarose gel. Transgene-positive mice were confirmed by Southern blotting. From the cDNA injections, 22 transgenic founder mice were generated and twelve of the founders were bred to generate females for this study. From the co-injection, 19 transgenic founders were identified, of which eight contained all three transgenes.

The introduction of specific oligonucleotides into the transcribed region of the transgenes permitted us to distinguish endogenous WAP transcripts ftom transgene transcripts. Total RNA was isolated (Chomczynski *et al.*, 1987) and duplicate samples of 10 μg were electrophoresed in 1.5% agarose formaldehyde gels and blotted onto GeneScreenPlus. Each blot was used for two oligonucleotide probings with stripping in between. Oligonucleotides were labelled using γ^{32}P ATP and T4 polynucleotide kinase. To detect endogenous WAP RNA, blots were probed with oligo 101 (5′ CAACGCATGGTACCGGTGTCA 3′) at 55°C. Transcripts from the genomic transgene was detected with oligo 102b (5′ GGCAACGCATGCAAGCTTGCGGTGTCAGGCA 3′) at 60°C. WAP-delta 3 transcripts were detected by hybridizing at 55°C with oligo 190 (5′ CGCATGGGATATCCCGGTGTC 3′). WAP-cDNA transcripts were detected by hybridizing at 55°C with oligo 192 (5′ CGCATGGCGGCCGCGTGTCAG 3′). Hybridization signals on northern blots were quantitated using a Betagen Betascope 603. As a control for hybridization efficiency, a synthetic RNA template was constructed, which contained

one copy of each of the oligonucleotide sequences. Since the different transgene sequences and the endogenous WAP sequence overlapping the *Kpn* I site were present in equal ratios in this synthetic RNA, corrections could be made for differences in the hybridization signal between the individual oligonucleotides.

Quantitation of transgene alleles

The copy number of the different transgenes was evaluated through the quantitation of the hybridization signals on Southern blots. In addition, the relative copy number of the WAP-genomic verses WAP-delta 3 was determined using the PCR Mimic procedure (Gilliand *et al.*, 1990). Mimic competitor DNA was prepared by amplifying exon 1 of nontransgenic genomic DNA using primers P1 and P2. The mimic template was therefore identical to the endogenous WAP gene. Two-fold serial dilutions of mimic DNA were added to a constant amount of transgenic genomic DNA in separate tubes for each of the three lines and the mixtures were amplified for 25 cycles, using primers P1 and P2 in a final reaction volume of 100 μl. Cycle conditions were 94°C, one minute, 60°C, one minute, 72°C, one minute. Each dilution series was divided into three sets containing 25 μl of PCR product. The first set was digested with *Hin* dIII, the second was digested with *Eco* RV and the third was digested with *Kpn* I. The digests were electrophoresed in 2% agarose TBE gels and briefly stained with EtBr to minimize EtBr background. The restricted fragments of each set of serial dilutions was then quantitated using the Collage™ program and a Fotodyne Foto/Analyst™ system. The fluorescent intensity of each band was plotted versus the mimic dilution. The relative copy number was then determined by extrapolating to the dilution axis from the point where each transgene intersected the mimic curve.

Results and discussion

Analysis of mice carrying only WAP cDNA transgenes

A 2.4 kb WAP gene promoter fragment and 1.6 kb of 3′ flanking sequence are sufficient to obtain expression of a WAP-genomic transgene in approximately 50% of the lines (Burdon *et al.*, 1991; McKnight *et al.*, 1992). To test whether these sequences can promote efficient expression of a WAP cDNA, we deleted all introns from a WAP-genomic transgene (Fig. 1). Twelve founder mice were generated which carried between two and 20 copies of the transgene. Lines were established, and mammary tissue from lactating females was analysed for the presence of transgenic WAP RNA (Fig. 2). No transgenic WAP RNA was detected in mice from nine lines, and three lines expressed the WAP transgene at a level of 0.02% or less of the endogenous gene (Fig. 2B). Mice from all 12 lines expressed the endogenous WAP gene at a high level (Fig. 2A).

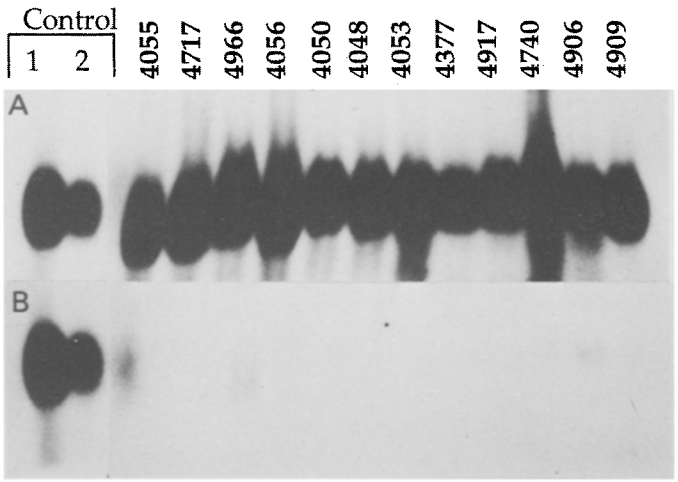

Fig. 2. Steady state levels of transgenic WAP RNA from mice from 12 lines carrying WAP-cDNA transgenes. Mammary RNA from lactating mice was separated in a formaldehyde gel, transferred onto GeneScreenPlus membrane and successively hybridized with oligonucleotide probes specific for the endogenous WAP gene (A) and the WAP-cDNA transgene (B). As a hybridization and exposure control, two dilutions of a synthetic RNA transcript, containing oligonucleotides specific to the WAP-cDNA transgene and the endogenous WAP gene, were included in the gel (control 1 and 2).

Analysis of mice carrying juxtaposed WAP cDNA and WAP genomic transgenes

The activation of a cDNA by a co-injected genomic clone could be due to enhancers within one or more introns. In order to determine whether any of the three introns of the WAP gene were required for high-level expression of a WAP-based transgene, it would be necessary to make constructs with all possible combinations of introns deleted. This would require generating a considerable number of lines. We therefore assumed that there would not be any positive effect and co-injected three different constructs at a time. If there were no interaction between the transgenes, the WAP-genomic transgene would express as previously reported (Burdon *et al.*, 1991; McKnight *et al.*, 1992) while the WAP-cDNA would remain silent as shown in this study. The particular significance of an intron(s) would determine whether an intron deleted transgene would be expressed more like the genomic or the cDNA. From the work of Hansson *et al.* (1994), it was clear that WAP introns 1 and 2 were not required: therefore, we chose to limit our co-injection analysis to the WAP-genomic, WAP-cDNA and WAP-delta 3 transgenes.

From the co-injections, eight founder mice which

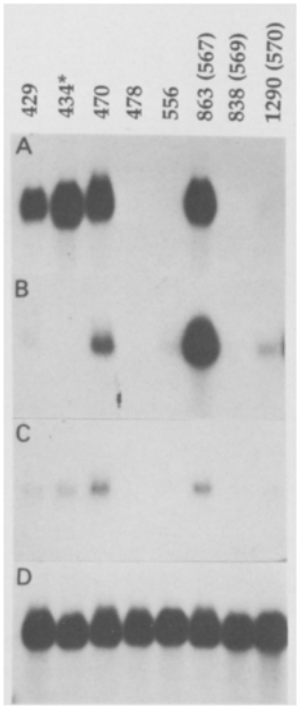

Fig. 3. Steady state levels of transgenic mice WAP RNA from mice carrying all three transgenes. Mammary RNA from lactating mice was separated in a formaldehyde gel, transferred onto GeneScreen and successively hybridized with oligonucleotide probes specific for the WAP-genomic (A), the WAP-delta 3 (B) or the WAP-cDNA (C) transgene. The hybridization signal for the endogenous WAP gene is shown in (D).

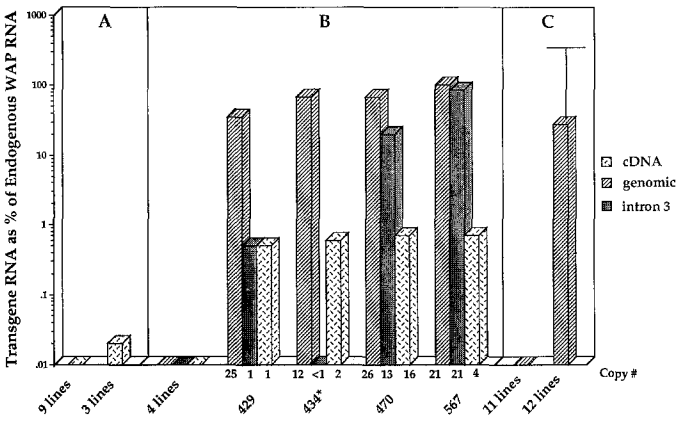

Fig. 4. WAP transgene expression as a percentage of endogenous WAP RNA. Panel A, lines containing the WAP-cDNA transgene, nine nonexpressing lines and three expressing lines. Values are presented as log values in order to illustrate differences between no expression versus minimal expression and allow for comparisons with panel B. Panel B, co-injected lines containing all three transgenes, four non-expressing and four expressing lines; the copy number of each transgene is listed below each bar. Panel C, lines containing the WAP-genomic transgene, 11 non-expressing lines and 12 expressing lines summarized from previous studies (Burdon *et al.*, 1991; McKnight *et al.*, 1992).

carried all three transgenes were identified. Lines were established from the three male founder mice (567, 569 and 570), and the co-segregation of all three transgenes strongly suggested that they had co-integrated in one locus. This was in agreement with earlier studies which had shown that co-injection of different DNA fragments resulted in co-integration of the fragments (Burdon *et al.*, 1991; Clark *et al.*, 1992; McKnight *et al.*, 1992). Transgene expression was analysed in mammary tissue from lactating females representing the three lines and in mammary tissue from the five female founder mice (Fig. 3). Expression of the WAP-genomic transgenes in three founder mice (429, 434 and 470) and in mice from one line (567) exceeded 35% of the endogenous WAP gene (Fig. 3A and Fig. 4B) which is in agreement with earlier studies (Fig. 4C; Burdon *et al.*, 1991; McKnight *et al.*, 1992). These same lines also expressed the WAP-cDNA transgene at levels approaching 1% of the endogenous WAP. Four other lines (478, 556, 569 and 570) which did not express the WAP-cDNA, also did not express the WAP-genomic transgene (Fig. 3 and Fig. 4B).

High levels of WAP-delta 3 RNA were detected in founder 470 and in line 567 (Fig. 3B and Fig. 4B) while very little expression was observed from founders 429 and 434. Southern analysis of founder 434 indicated that the WAP-delta 3 transgene was present at less than one copy and therefore mosaic.

The copy number of the different co-injected transgenes in the expressing animals was determined by quantitative Southern blot and PCR mimic analyses. The number of integrated copies was variable and ranged from 12 to 26 for WAP-genomic, one to 21 for WAP-delta 3, and 1 to 16 for WAP-cDNA (Fig. 4B). The steady state RNA level was highest for the WAP-genomic construct in all expressing lines. However when transgene expression per copy is compared in lines 429, 470 and 567, the WAP-genomic and WAP-delta 3 transgenes are equivalently expressed at approximately 1, 2 and 4% of endogenous WAP respectively. Therefore, within a line the two transgenes showed copy-number-dependent expression, which supports the previous finding by Burdon *et al.* (1991). From these results and the work of Hansson *et al.* (1994), it appears that a WAP-based transgene does not require a WAP-specific intron to promote transgene expression from a WAP promoter-based construct.

When the steady state RNA levels of the WAP-cDNA from the co-injection were compared to those of the singly injected WAP-cDNA, it appeared that some enhanced expression occurred whenever the WAP-cDNA co-integrated with a WAP-genomic transgene into an expressing locus (compare Fig. 4B with 4A). The expression levels are presented using log values in order to be able to show the difference between the weakly expressed single and co-injected WAP-cDNAs and the much more highly expressed genomic constructs. The WAP-cDNA expres-

sion compared to WAP-genomic and WAP-delta 3 or the endogenous WAP was quite low (compare WAP-cDNA expression in Fig. 4A and Fig. 4B to the WAP-genomic transgene expression in Fig. 4B, co-injection, and WAP-genomic single injection, Fig. 4C). This basal level of expression is more than two orders of magnitude below the endogenous WAP potential and may not reflect expression from a bona fide WAP transcription complex. Similar results were also found by Clark *et al.* (1992). RNA levels from an α1-antitrypsin cDNA transgene were in many cases three orders of magnitude lower than those from the juxtaposed sheep β-lactoglobulin transgene.

Influence of co-integrated genomic transgenes on cDNA transgenes

Activation of cDNA-based transgenes has been achieved through the inclusion of homologous or heterologous introns (Choi *et al.*, 1991; Palmiter *et al.*, 1991). In these cases however, increased RNA levels may have reflected the presence of *cis*-acting transcription elements within introns (Whitelaw *et al.*, 1991; Liska *et al.*, 1994). The activation mechanism seen here is not likely simply due to an intron based enhancer effect because transcription elements necessary for high level WAP gene expression appear to be within the promoter region. Paleyanda *et al.* (1994) used the WAP gene promoter without WAP introns to express a genomic protein C transgene at high levels (Paleyanda *et al.* 1994). Hansson *et al.* (1994) expressed SOD with a WAP-based construct in which WAP introns 1 and 2 were deleted, and, in this study, intron 3 was not required for WAP transgene expression.

The presence of introns might contribute to activation by helping to promote or maintain open chromatin. However, our results suggest that if such an effect was occurring here, it was minimal and only allowed for basal levels of transcription and may not have resulted in the assembly of complete WAP-specific transcriptional complexes onto the WAP-cDNA transgene. It is also necessary to consider post-transcriptional events such as splicing, in the chain of transgene activation. The absence of splicing has been shown to influence 3′ processing and polyadenylation and resulted in inefficient transport of the mature mRNA from the nucleus (Huang and Gorman, 1990).

In conclusion, we have shown here that the co-injection of genomic sequences containing promoter specific introns along with hybrid transgenes containing cDNA's does not result in significant activation of a silent cDNA. These results caution against the general application of activating silent cDNAs through co-injection of genomic sequences.

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