The kinetics of rAAV integration in the liver

Recombinant AAV vectors have been developed which lack all viral coding sequences, thus eliminating the potential of immune responses toward viral proteins[1]. These vectors can effectively transduce non-dividing cells, including neurons, skeletal muscle and hepatocytes, and persist long-term *in vivo*[2–5]. We reported recently that rAAV-mediated transduction of mouse livers (*via* intraportal infusion) with human factor IX gene (*F9*) results in up to 2000 ng/ml of fully functional human factor IX which persisted for almost a year[4]. A slow rise over several weeks in transgene-originated protein was observed in muscle[5–8], liver[4], retina[9] and subcutaneous tissues (B.D. *et al.*, unpublished results) after vector administration. Southern-blot and PCR analyses in muscle indicate a mechanism for transgene persistence by the conversion of the monomer genomes to a high-molecular-weight state, but the integration status of the vector into the host genome was not confirmed[5,7,10].

To determine whether the slow rise in serum factor IX levels over several weeks correlates with a change in the rAAV genomes in hepatocytes, mice were infused with 4.8×10^{10} particles of rAAV-MFG-hF9 (ref. 4), and subsequently examined at different time points. A Southern blot of undigested genomic DNA extracted from mouse livers one day after gene transfer revealed single-stranded vector genome (Fig. 1*a*, lanes 6–8) that slowly decreased for 5 weeks and was undetectable by 13 weeks (Fig. 1*a*, lanes 18–20). Digestion of these samples with *Bam*HI/*Nhe*I (Fig. 1*c*) or *Sph*I/*Nhe*I (Fig. 1*e*, lane 12) releases internal fragments of 3.2 or 3.5 kb, respectively, containing the *F9* cDNA (Fig. 1*b*, lanes 1–4). A Southern blot of the digested samples showed faint signals of the appropriate size one week after rAAV instillation into mouse liver; these gradually increased to a steady-state level at five weeks (Fig. 1*c*). The increase in double-stranded rAAV DNA was proportional to the loss of single-stranded DNA. Digestion of these samples with *Bam*HI, which digests the vector once near the 3′ end, yielded a major band of approximately 3.9 kb at 5 weeks and 13 weeks after gene transfer (Fig. 1*b*, lanes 6–20 and 1*e*, lane 11). Collectively, these

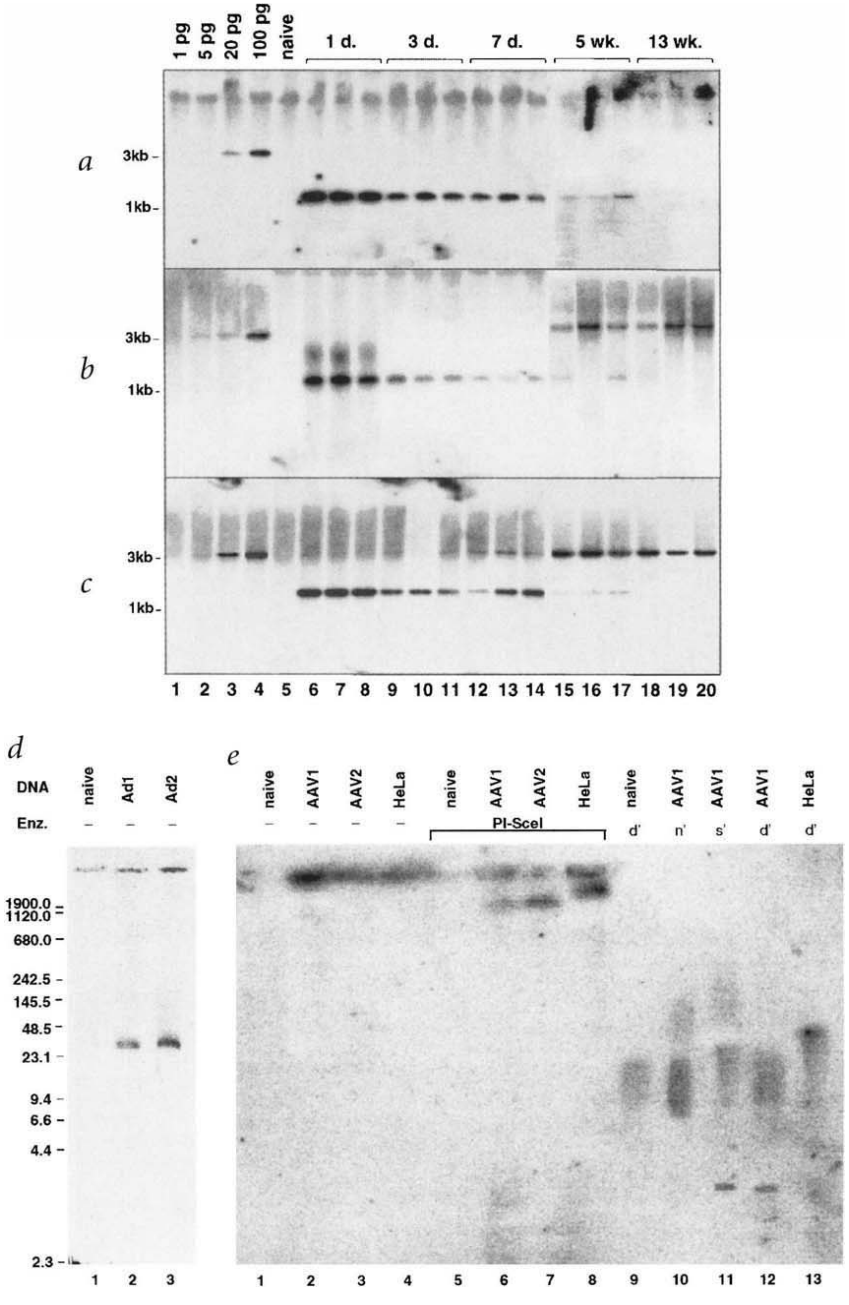

Fig. 1 Southern and pulse-field analyses. ***a–c***, Total cellular DNA was isolated from the livers of mice. DNA from naive mice with or without added pSSV9-MFG-hF9 plasmid DNA was used as positive and negative controls. DNA samples (10 µg) were left undigested (*a*); digested with *Bam*HI (*b*); digested with *Nhe*I and *Bam*HI (*c*) and analysed by Southern blot. Hybridization was with a ^{32}P-radiolabelled 810-nt *F9* fragment extending from exon II to exon VIII of the *F9* cDNA. Lanes 1–4: 1, 5, 20, 100 pg of pSSV9-MFG-hF9 plasmid mixed with genomic DNA isolated from a naive mouse. Lane 5, liver DNA from a naive mouse. Lanes 6–20, liver DNA from AAV-treated mice examined at different time points: lanes 6–8, day 1; lanes 9–11, day 3; lanes 12–14, day 7; lanes 15–17, week 5; lanes 18–20, week 13. ***d–e***, Agarose plugs[11] were made from primary mouse hepatocytes by perfusion with collagenase[12]. Analytical pulse-field gel electrophoresis was performed with the undigested or digested plugs as previously described[11]. (*d*) Control experiment. Hepatocyte plugs from naive mouse (lane 1) or mice treated with adenoviral vectors encoding canine factor IX (lanes 2 and 3). The probe contained the complete coding region of canine *F9* cDNA. (*e*) AAV experiment. Agarose plugs from naive or AAV-treated mouse livers at 13 weeks after rAAV transduction, or HeLa cells. Untreated plugs: Naive mouse (lane 1); AAV-treated mice (lane 2 & 3); HeLa cells (lane 4). Endonuclease PI-SceI digested plugs: Naive mouse (lane 5); AAV-treated mice (lanes 6 and 7); HeLa cells (lane 8). *Nhe*I/*Sph*I digested plugs: Naive mouse (lane 9); AAV-treated mice (lane 12); HeLa cells (lane 13). Plugs from AAV-treated mice, digested with *Hind*III (lane 10) or *Bam*HI (lane 11).

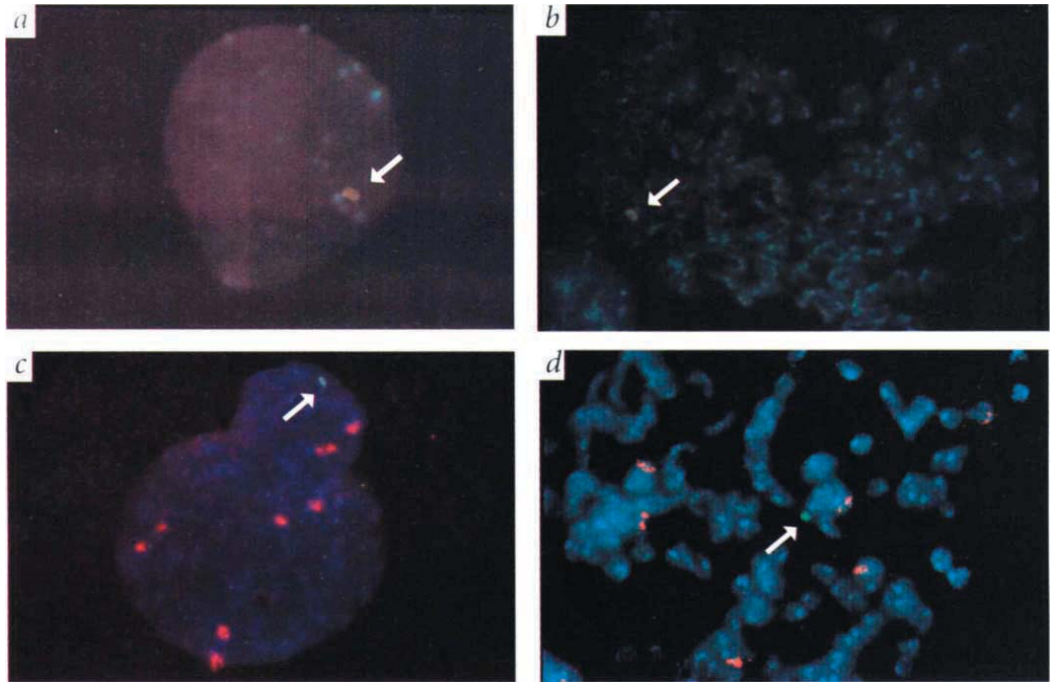

Fig. 2 Fluorescent *in situ* hybridization of AAV-hF9. Metaphase-containing nuclei were prepared from hepatocytes from normal and AAV-treated mice, cultured in William's medium containing 10% fetal calf serum and 20 ng/ml EGF (ref. 13) for 40 h. Mouse interphase nuclei from freshly isolated primary hepatocytes, and metaphase spreads from the cultured hepatocytes for FISH were prepared by a standard methanol-acetic acid protocol. An X-chromosomal probe which contains a 9-kb insert hybridizing to a centromeric repeat sequence[14] (n=20) was labelled by nick translation, incorporating biotin-14-dATP (Life Technologies), and the AAV probe made from the rAAV plasmid, pSSV9-MFG-hF9 was labelled by nick translation, incorporating Digoxigenin-11-dUTP (Boehringer) prior to hybridization[15]. Texas Red-conjugated avidin in combination with biotinylated goat anti-avidin were used for detection of hybridization signals generated from the X-chromosomal probe. Mouse anti-Digoxigenin in combination with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG were used to detect hybridization signals generated from the AAV probe. Slides hybridized with AAV probe only: (***a***) Interphase nuclei, (***b***) Metaphase chromosomes; slides hybridized with both X-chromosomal probe and AAV probe: (***c***) Interphase nuclei, (***d***) Metaphase chromosomes. The chromosomal DNA was identified by the blue 4,6-diamidino-2-phenylindole (DAPI) stain. The discrete green hybridization signals were produced by hybridizing with a specific AAV vector probe, the red signals from hybridizing with an unique X-chromosomal probe.

data indicate that the single-stranded vector genomes were converted to double stranded head-to-tail concatemers in hepatocytes over a period of five weeks.

To determine if the rAAV was integrated, we used pulse-field gels to separate high-molecular–weight (10 Mb to 3 kb) concatemerized episomal vector forms from chromosomal DNA. The undigested DNA from rAAV-transduced hepatocytes had an *F9* signal that comigrated with chromosomal DNA just below the well of the gel. However, no episomal forms of the vector DNA were detected (Fig. 1*e*, lanes 2 and 3). The absence of a signal in the sample prepared from a naive mouse (Fig. 1*e*, lane 1), and a signal in HeLa cells (Fig. 1*e*, lane 4), confirmed the specificity of the human probe. Two experiments confirmed that a high-molecular–weight episome, if present, was not trapped within the chromosome. First, a 35-kb adenoviral genome was clearly separated from mouse chromosomal DNA in analyses of control animals infused with adenovirus (Fig. 1*d*, lanes 2 and 3). Second, the DNA samples were treated with endonuclease PI-*Sce*I which cut the chromosomal DNA into fragments that were mostly greater than 1 Mb as confirmed by ethidium bromide staining (not shown). The rAAV signal migrated into the region of the gel corresponding to a size that was greater than one Mb (Fig. 1, lanes 5 and 6).

Based on our previous experiments, we estimated that there were approximately 3.5 rAAV genomic copies per transduced cell with gene expression detected in about 5% of hepatocytes[4], suggesting the average high-molecular–weight concatemers formed from the vector genome would have a molecular size of about 275 kb (70 copies of an approximately 3.9-kb monomer), assuming that most of the rAAV-containing cells express transgene product. Because we did not observe any episomal forms of rAAV vectors less than 1 Mb in size, and rAAV vector sequences were associated with high-molecular-weight genomic DNA, it appears that the rAAV proviral DNA was stably integrated into the mouse chromosome.

To further establish integration of rAAV in mouse hepatocyte chromosomes and determine the proportion of cells that contain rAAV double-stranded genomes, FISH analysis was performed on interphase nuclei and metaphase spreads prepared from mouse hepatocytes. Red signals generated from a unique X-chromosomal probe were detected in all of the nuclei prepared from normal and AAV-treated animals (Fig. 2*c,d*), sometimes in multiples because hepatocytes often contain polyploid nuclei. A discrete green signal generated from hybridization with a rAAV vector probe was detected in 58 of 1203 (4.8%) of interphase nuclei (Fig. 2*a,d*) and on sister chromatids in 12 of 215 (5.5%) of metaphase chromosomes (Fig. 2*b,d*). These results confirmed that rAAV genomes were randomly integrated into chromosomal DNA in transduced cells. The presence of vector DNA in about 5% of hepatocytes correlates with the number of hepatocytes shown previously to express transgene product[4], demonstrating that the majority of cells containing the double stranded concatemeric vector DNA were transcriptionally active.

The integration status of the rAAV strongly suggests that for hepatic gene transfer, gene expression can reach thera-

peutic levels and should be life-long and suitable for the treatment of genetic diseases. The mechanism which results in a relatively small percentage of hepatocytes containing multiple rAAV genomes will require further study. Perhaps a threshold amount of vector genome and/or the presence of an as yet unknown cellular milieu is required for integration.

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An imprinted antisense RNA overlaps *UBE3A* and a second maternally expressed transcript

Angelman syndrome (AS), characterized by a movement disorder, seizures, hyperactivity and severe mental retardation[1], is caused by the absence of a maternal contribution to the imprinted chromosome 15q11–q13 region. Although most cases are associated with abnormal methylation and/or chromosomal abnormality, a fourth class of AS patients has normal methylation and biparental contribution to the 15q11–q13 region. Mutations of the gene encoding ubiquitin protein ligase 3A (*UBE3A*) have been detected in this AS class[2,3], and it has been shown that *UBE3A* is expressed predominantly from the maternal chromosome 15 in brain[4,5]. Although these results support the causative role of *UBE3A* abnormalities in AS, the presence of *UBE3A* mutations in only 20–30% of the relevant class of patients suggests that other genes could be directly or indirectly involved in the pathogenesis of this disease.

To study *UBE3A* imprinting, we performed RT-PCR on normal, AS and PWS (Prader-Willi syndrome) brain samples, using random hexamers for the reverse transcription (RT) and primers located in exons 15 and 16 of *UBE3A* (3F and 3R) for PCR (Fig. 1*c*). In addition to the expected band of 152 bp which corresponds to the spliced *UBE3A* product, a second, larger product was detected (Fig. 1*a*), which was shown by sequencing to be the unspliced form of *UBE3A*. Surprisingly, whereas the spliced product is less abundant in AS brain (reflecting the maternal expression of *UBE3A*), the unspliced form is reduced in PWS brain, suggesting a preferential paternal expression of this splice variant (Fig.1*a*). Given the recent demonstration that the regulation of imprinted genes can involve an interaction between sense and antisense transcription[6], we performed RT of normal brain RNA using primers specific for each strand, followed by PCR with the 3F and 3R primers. The 'unspliced' product is observed only when the antisense specific primer is used, indicating that it is derived from an antisense transcript (Fig. 1*b*). Similar results were obtained using RT and PCR primers flanking exons 11 and 12 of *UBE3A* (data not shown), suggesting an antisense transcript for *UBE3A* spans at least the 3′-half of the gene. This antisense transcript displays a pattern of imprinting that is opposite to that of *UBE3A*, with preferential expression from the paternal allele in brain.

To characterize the antisense transcript, and especially its 5′ end, normal brain RNA was reversed transcribed (RT) using a series of antisense-specific primers from the 3′ end of *UBE3A* (intron 15) to a genomic region 6 kb downstream (primers E6Z-A, 10F, A8 and 55, Fig. 1*c*). The RT product was then amplified with primers 51 and 52, located approximately 6.5 kb from the *UBE3A* stop codon. A PCR product was obtained for each of these RT reactions, even for those using a primer in *UBE3A* intron 15, E6Z-A (data not shown). This indicates that the antisense transcript covers at least the 3′ half of *UBE3A* (from exon 11 to the 3′ end) and additional sequence downstream. 5′ RACE extended the sequence by only 60 bp, further suggesting that the 5′ end of the antisense transcript is 6.5 kb from the stop codon of *UBE3A*.

Because the antisense transcript extends downstream of *UBE3A*, we investigated the possiblity of a second 'sense' gene located 3′ of *UBE3A* by performing RT-PCR using primers specific for both strands. We were able to detect, in addition to the antisense transcript, a product in normal brain that corresponds to the sense strand (data not shown), and therefore has the same transcriptional orientation as *UBE3A*. Several rounds of 5′ RACE showed that the 5′ end of this downstream sense transcript is located between the second and third polyadenylation signals of *UBE3A* (ref. 7), suggesting that the downstream transcript promoter lies in the region corresponding to the 3′ UTR of *UBE3A* (Fig. 1*c*). Although this transcript remains to be fully characterized, our preliminary data indicate that it is intronless and spans approximately 3.5 kb (Fig. 1*c*, and data not shown). Another hypothesis that cannot be excluded, however, is that the downstream transcript is an alternative 3′-splice variant of *UBE3A*.

To determine whether the downstream transcript is imprinted, brain RNAs (normal, AS and PWS) were reverse-transcribed using a primer specific for the antisense (10F) and a second for the sense (e3R) strand. These primers flank the region amplified by primers 11F and e1R (Fig. 1*c*). As a control, the RNAs were reverse-transcribed using a primer specific for the dystrobrevin gene (primer HS86)