Targeting Genes: Delivery and Persistent Expression of a Foreign Gene Driven by Mammalian Regulatory Elements in Vivo*

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We present evidence that a foreign gene driven by natural mammalian regulatory elements can be targeted to hepatocytes and the resultant gene expression made to persist. This was accomplished using a soluble DNA carrier system consisting of two covalently linked components: 1) a polycation, poly-L-lysine, that can bind DNA in a strong but non-damaging interaction, and 2) an asialoglycoprotein which can be targeted specifically to hepatocytes by cell surface asialoglycoprotein receptors unique to this cell type.

A plasmid, pab-CAT, containing the gene for chloramphenicol acetyltransferase (CAT) driven by mouse albumin regulatory sequences was complexed to the carrier system. Intravenous injection of pab-CAT DNA in the form of a complex resulted in the presence of CAT enzyme activity in liver homogenates 24 h after injection. The targeted gene expression, however, was transient, reaching a maximum of 10 units/g liver at 24 h but was not detectable by 96 h. However, partial hepatectomy 30 min after injection resulted in persist-ent high levels of hepatic CAT activity (11.3 units/g) through 11 weeks post-injection. Southern analysis of livers 11 weeks after partial hepatectomy demonstrated that some of the targeted DNA had been integrated into the host genome. We conclude that a foreign gene driven by natural mammalian regulatory elements can be delivered to hepatocytes by intravenous injection in vivo using a soluble DNA carrier system. Foreign gene expression targeted in this manner can be made to persist by stimulation of hepatocyte replication.

We have previously shown that a foreign gene complexed to a soluble DNA carrier system can be targeted and expressed in hepatocytes in vivo (1). In that study the DNA consisted of a plasmid, pSV2 CAT, containing the gene for chloramphenicol acetyltransferase (CAT) driven by an SV40 viral promoter. We wondered whether a foreign gene driven by natural mammalian regulatory sequences could similarly be targeted and expressed and, if so, whether the targeted foreign gene expression could be made to persist.

To test these possibilities, we used DNA in the form of a plasmid, pab-CAT, which contains the CAT gene driven by mouse albumin regulatory elements. We based our experiments on the following facts: 1) normal hepatocytes possess unique cell surface receptors that can recognize and internalize galactose-terminal (asialo)glycoproteins (2) ; 2) polycations, e.g., poly-L-lysine, can bind DNA in a strong, non-damaging interaction to form soluble complexes (3); 3) CAT is a bacterial enzyme that can act on chloramphenicol to produce acetylated derivatives. Because the CAT gene is absent in mammalian cells, the presence of CAT activity can serve as a convenient marker for targeted gene expression (4).

MATERIALS AND METHODS

Preparation of a Targetable DNA Carrier—To form a carrier system capable of being targeted specifically to hepatocytes, orosomucoid was isolated from pooled human serum (American Red Cross, Farmington, CT) (5) and desialylated with insolubilized neuraminidase (Type X-A, Sigma) to form asialoorosomucoid (AsOR) (6). Residual sialic acid was determined to be less than 10% (7). Poly-L-lysine, mean M, = 3,800 (Sigma), was coupled to AsOR in a 2:1 molar ratio using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce Chemical Co.) adapted from the method of Halloran and Parker (8), purified, and analyzed as described previously (9).

Plasmid Preparation—The pab-CAT construct was prepared by replacement of an SV40 early promoter by mouse albumin promoter (~350 to +10 base pairs) and enhancer sequences (~12 to ~35 kilobases) (10) in the plasmid MTBV.JT. The plasmid was cloned in Escherichia coli, isolated, and purified (11). Purity was confirmed by 1% agarose gel electrophoresis demonstrating the absence of bacterial cellular DNA. For hybridization studies, the CAT insert was labeled with 32P by nick translation (12).

Formation of a Targetable Carrier-DNA Complex—The optimal proportions for complex formation between the pab-CAT plasmid and the AsOR-poly-L-lysine conjugate were determined using an agarose gel retardation system as described previously (13). A conjugate to DNA molar ratio of 25:1 (based on AsOR content of the AsOR-poly-L-lysine conjugate) was found to completely retard DNA migration in the gel and form soluble complexes, and this ratio was used in all subsequent experiments. All complexes were filtered through 0.45 μm membranes (Millipore) prior to injection to ensure that samples used did not contain precipitates. Complexes were found to be stable in saline or in rat serum at 37°C for at least 1 h, and in saline at 4°C for at least 2 weeks.

Targeted Gene Expression—To assess targeted gene expression, female Sprague-Dawley rats (220-250 g) in groups of two were injected intravenously with 1 ml of saline containing 580 μg of pab-CAT DNA in the form of AsOR-poly-L-lysine-DNA complex or controls, and at daily intervals animals were killed and liver samples were removed and homogenized. Homogenates were assayed for protein content (14) and equal amounts of homogenate protein assayed for CAT activity (4).

To examine the effect of partial hepatectomy on the time course of targeted gene expression, groups of rats were injected intravenously with AsOR-poly-L-lysine-DNA complex, and 30 min later 65% partial hepatectomies were performed (15). Animals were maintained over

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‡ The abbreviations used are: CAT, chloramphenicol acetyltransferase; AsOR, asialoorosomucoid.
an 8-week period during which they were periodically killed, livers removed and homogenized, and hepatic CAT activity determined. At the 8-week point, a lobectomy was performed and animals killed at 11 weeks. All CAT assays were performed in duplicate.

State of the Targeted DNA Associated with Persistent Gene Expression—To determine the state of the DNA targeted by our delivery system, DNA was extracted from liver homogenates by a phenol/chloroform method (16). Samples of DNA were digested with BstEII (2 units/μg DNA) which does not cut the palb-CAT plasmid, XhoI (2 units/μg DNA) which cuts the plasmid at a single site, and BamHI (2 units/μg DNA) which excises the CAT insert from the plasmid. All digestions were carried out at 37°C for 18 h after which time samples were applied in increasing concentrations on an 1% agarose gel for electrophoresis along with palb-CAT plasmid. The DNA was then transferred to nitrocellulose and CAT sequences detected by hybridization with a 32P-labeled CAT cDNA probe (17).

RESULTS AND DISCUSSION

Targeted foreign gene expression as a function of time is shown in Fig. 1, a representative assay for CAT gene expression. CAT activity was 10 units/g liver at 24 h and 7.6 units/g 48 h after injection. However, the expression was transient as activity declined to 4.6 units/g at 72 h, and by 96 h CAT activity was no longer detectable.

The effect of 66% partial hepatectomy on the time course of hepatic targeted gene expression is shown in Fig. 2. Lane 3 shows that CAT activity was not detectable 24 h after hepatectomy but was restored by 48 h to a level of 2.4 units/g liver. This activity remained detectable well beyond the 96-h limit seen in the transient studies shown previously. CAT activity actually increased to a maximum of 14.6 units/g liver by the 8th week and persisted at high levels, 11.3 units/g, through the 11th week post-hepatectomy.

To determine the state of the targeted DNA in livers with persistent CAT gene expression, DNA was extracted from portions of DNA complex-treated livers 11 weeks post-partial hepatectomy. In Fig. 3, lanes 1–3 contain palb-CAT plasmid, 0.01, 0.05, and 0.1 μg, respectively, linearized by digestion with XhoI which cuts the plasmid at a single site. Lane 4 shows the electrophoretic position of the CAT insert excised from the standard palb-CAT plasmid by BamHI. Lane 5 shows that cellular DNA from the livers treated with the targetable DNA complex and analyzed 11 weeks after partial hepatectomy contained high molecular weight sequences that hybridized with the CAT cDNA probe. Digestion of this cellular DNA with BamHI, shown in lane 6, resulted in complete release of the CAT insert which migrated in a manner identical to the insert excised from standard palb-CAT plasmid (lane 4). Lanes 7–9 show that XhoI digestion of cellular DNA from the livers treated with complex and analyzed 11 weeks post-partial hepatectomy resulted in the formation of some hybridizable fragments of lower molecular weight than the intact linear plasmid, but the majority of the hybridizable sequences remained present as DNA greater in size than the linear form of the plasmid. Restriction of cellular
Targeting Genes in Vivo

DNA by an enzyme that does not cut the plasmid, BstEII, shown in lanes 10–12, resulted in the formation of hybridizable fragments that were all greater in size than the parental CAT plasmid. Doubling the ratios of restriction enzymes to cellular DNA as well as the duration of digestion with XbaI and BstEII did not change the restriction patterns (data not shown). The copy number in samples of livers 11 weeks post-transfection was calculated to be approximately 18.1. Finally, lane 13 shows that cellular DNA from control livers (saline-treated) analyzed 11 weeks after partial hepatectomy demonstrated no hybridizable CAT sequences, indicating that the observed hybridization by complex-treated liver DNA was not due to nonspecific binding of the probe to any endogenous host sequences.

In our previous studies we have shown that a foreign gene driven by an SV40 viral promoter can be targeted to, and expressed in, hepatocytes in vivo (1). Our current data indicate that genes controlled by natural, mammalian regulatory elements can be similarly delivered and expressed in vivo.

Because the CAT marker gene used in our current studies is controlled by mouse albumin regulatory elements, CAT expression would be expected to reflect the normal regulation of albumin synthesis in the targeted cells. It has been shown previously by Petropoulos et al. (18) that albumin mRNA levels remain essentially unchanged after partial hepatectomy in young (120–150 g) rats. However, hepatic regeneration induced by CCl4 was found by Panduro et al. (19) to result in a decrease in hepatic albumin mRNA levels at 24 h followed by a rapid recovery. In experiments more closely related to the currently described work, Pricen et al. (20) found that partial hepatectomy in rats 250–300 g in size resulted in a decline in albumin mRNA to very low levels at 12 h. Levels remained low until 48 h after surgery (20). The latter effects on the albumin mRNA levels were not specifically due to the partial hepatectomy but more than likely represented a response of older rats to surgery in general. Nevertheless, this pattern is very similar to that seen with our targeted CAT gene expression (Fig. 2) in rats of a similar age and size (220–250 g). The data suggest that the albumin regulatory sequences of the targeted CAT gene functioned in a manner that reflects the normal control of albumin synthesis in hepatocytes in vivo.

Previous investigators have shown that persistence of foreign gene expression is enhanced when exogenous DNA is introduced while host DNA replication is in progress in vitro (21). The fact that partial hepatectomy resulted in foreign gene expression 11 weeks post-surgery indicates that the capability of hepatocytes to replicate in response to injury may be used to advantage to achieve persistence of targeted gene expression in vivo. In this regard, a mechanism by which persistence was achieved is suggested by the fact that high molecular weight hybridizable DNA was still present after restriction of cellular DNA with XbaI. Because this enzyme cuts within the plasmid at a single location, the finding of high molecular weight hybridizable sequences after this digestion indicates that simple tandem head-to-tail multimeric forms of intact plasmid could not account for the observed hybridization with unrestricted cellular DNA (Fig. 3, lane 5). Rather, the data support the notion that integration of the targeted CAT gene into the host genome has occurred. The lack of smears of hybridizable DNA after restriction was unexpected and suggests that the integration events that followed targeted gene delivery and hepatocyte replication occurred in a nonrandom manner. The mechanism by which this occurs is currently under investigation. Alternatively, but less likely, the tissue samples may have contained a limited number of clones that possessed unique integration sites.

Finally, in view of the exclusive hepatic specificity of the mouse albumin regulatory elements employed in our plasmid (10), the presence of CAT gene expression in liver after administration of the targetable DNA complex confirms our previous data that the soluble DNA carrier system does, in fact, target the foreign (CAT) gene to hepatocytes.

We conclude that a foreign gene driven by natural mammalian regulatory elements can be delivered to hepatocytes by intravenous injection in vivo using a soluble DNA carrier system. Furthermore, the resultant foreign gene expression can be made to persist by stimulation of hepatocyte replication during liver regeneration. This system offers exciting prospects for the study of gene regulation in vivo and may be of value in the study of somatic cell gene replacement therapy in models of inherited metabolic disorders.

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