Proc. Indian natn Sci Acad.(PINSA) **B68No.4** pp 315-332 (2002)

Targeted Delivery of Genes

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(Received on 23 April 2001; Accepted after revision 30 August 2001)

In the past few years, various gene delivery vehicles have been designed in order to make a vector effective for gene therapy application. Both viral and non-viral vectors have been used extensively as biological carriers for the transfer of foreign genes into living cells. But these vectors suffer from several draw backs which limit there use in future clinical application while using "gene therapy" for the treatment of several inherited disorders. The reconstituted Sendai viral envelopes, also known as virosomes are able to successfully deliver genes and other biologically active macromolecules to various cells in culture. Although such virosomes are known to fuse efficiently with the plasma membrane of target cells and served as excellent carriers for the fusion-mediated transfer on DNA, RNA, toxins, drugs, etc. into viable cultured cells, they result in non-specific delivery because of the presence of hemagglutinin protein (HN) and hence can not be used *in vivo*. On the other hand, reconstituted Sendai viral envelope devoid of the HN protein (F-virosomes) can fuse efficiently and preferentially with liver cells (both *in vitro* and *in vivo*) and deliver its contents to the cytosol of these cells. We conclude that this kind of targeted delivery can further be used for a wide variety of gene transfer strategies both *in vitro* and *in vivo* in the field of "gene therapy".

Key Words: Viral Vectors, Liposomes, Virosomes, Gene Delivery, Tissue Targetting, Cell Type-Specific Gene Delivery, Gene Expression

Introduction

In 1990, the first federally approved clinical trials for treatment of a genetic disorder by "gene therapy" began under the leadership of R Michael Blease, W French Anderson, and their colleagues at the National Institutes of Health. This technique involves the identification of required DNA sequences and cell types followed by appropriate ways of DNA delivery to the diseased cells. The therapeutic potential ranges from treating of genetic defects and slowing the progression of tumors, to fighting viral infections and curing neurodegenerative disorders. A unique set of problems, such as the lack of efficient delivery systems, lack of sustained expression, and host immune reactions still put forward formidable challenges. It has been almost unanimously pointed out that the major constraint in the field of "gene therapy" is to devise ideal 'vehicles' to target appropriate cells and achieve physiological levels of the desired gene product (Verma & Somia 1997). Several new strategies have been presented in the recent past for correction of genetic diseases of muscle and skin. Encouraging reports have been published in delivering genes encoding liver-

Abbreviations: *MuLV*, Murine leukemia virus; *MoMLV*, Moloney murine leukemia virus; *EPO*, Erythropoietin; *LDLR*, Low density lipoprotein receptor; *EGF*, Epidermal growth factor; *ASGPR*, Asialoglycoprotein receptor; *VSV*, Vesicular stomatis virus; *BUGT 1*,Bilirubin; *UDP*, glucuronosyltransferase; *RES*, Reticuloendothelial system; *F*, Fusion glycoprotein of Sendai virus; *HN*, Hemagglutinin-neuraminidase glycoprotein of Sendai virus; *RBC*, Red blood cells; *WGA*, Wheat germ agglutinin; *FCS*, Fetal calf serum; *PBS*, Phosphate-buffered saline; *TX-100*,Triton X-100; *DTT*, Dithiothreitol; *CAT*, Chloramphenicol acetyl transferase

derived factor IX to correct hemophilia B and fumarylacetoacetate hydrolase to cure hereditary tyrosinemia type I. Serious attempts to target genes to precise locations (such as airway epithelial cells of the respiratory tract) in diseases like "cystic fibrosis" have so far proved to be difficult and exemplify the need for new delivery methods. Newer viral and non-viral vectors/vehicles, which hold promise to facilitate target-specific vector uptake and retention and are able to evade unwanted immune responses, constitute the major demanding criteria for durable and successful gene therapy for genetic diseases (Blau & Khavari 1997).

It is understood that improving in the accuracy of a vehicle often compromises its efficiency and vice versa. Nevertheless, it is important to note that the technology now available for specific targeting encompasses most of the currently available delivery systems. The choice is either at the level of (a) target cell surface recognition, by exploiting the strong ligand-receptor interaction of viruses and liposomes or (b), by incorporating transcriptional elements into plasmid (expression vectors) or viral genomes in such a way so as to trigger the expression of the therapeutic gene only in target cell types of interest. In a nutshell, this may require an appropriate amalgamation of the most opted features of a variety of viral and nonviral vectors into a single "hybrid" carrier (figure 1) selectively custom-made for each individual therapeutic condition (Miller & Vile 1995). Technical detail of such a "hybrid vector" as we have developed in our laboratory for liver-directed gene transfer *in vivo* is being presented here.

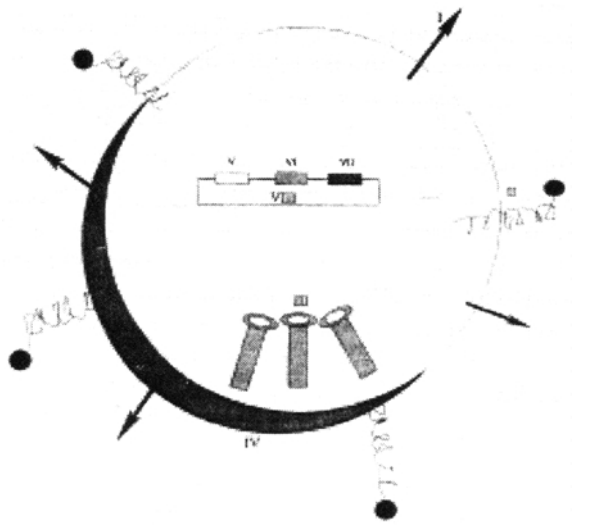

Figure 1 Hypothetical targeted gene courrier I, specific ligands; II, membrane fusogen; III, factor for directed integration of vector DNA (Site-specific recombinanase); IV, membranous envelope; V, sequence for specific homologous recombination; VI, tissue specific promoter; VII, therapeutic gene; VIII, cDNA vector

A Survey of Various Targeted Gene Transfer Protocols

Retroviral Vectors

Retroviruses are enveloped RNA viruses that replicate through a DNA intermediate synthesized by a viral enzyme, reverse transcriptase. The parental virus attaches to a susceptible cell through a specific receptor and enters the cell by a membrane fusion mediated process (figure 2). These RNA viruses are capable of mediating efficient gene transfer into many cell types and can stably integrate into the host cell genome,

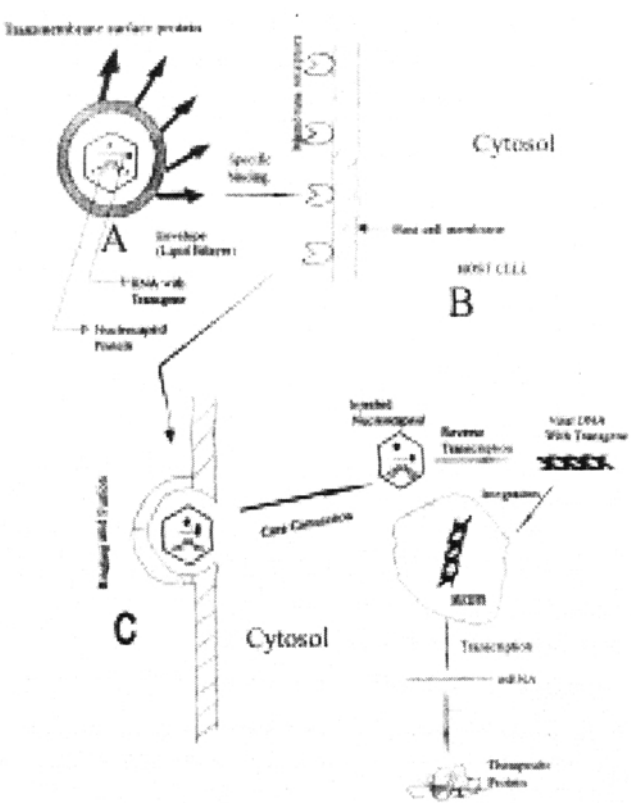

Figure 2 General entry mechanism of retroviral vectors

providing thereby the possibility of long term gene expression. With the exception of human immunodeficiency viruses (HIV) and human T-cell lymphotropic viruses (HTLV), the retroviruses bear minimal risk since they have evolved into relatively non-pathogenic viruses. Murine leukemia virus (MuLV) has traditionally been used as the vector of choice in some clinical gene therapy protocols. Various packaging systems to enclose the vector genome within such viral particles have also been developed. These vectors devoid of all viral genes are fully replication-defective and can encapsulate approximately 8Kb of exogenous DNA.

The major problems faced in developing retroviral vectors that are effective in treating disease are of four main types: (i) obtaining targeted and efficient delivery; (ii) transducing non-dividing cells; (iii) sustaining long-term gene expression; and (iv) developing a cost-effective way to manufacture the vector (Verma & Somia 1997).

Major efforts have been centered on attempts to engineer the natural retroviral envelope protein with a view to generate its cell type specificity. The envelope protein is endowed with two functions: binding to its receptor (by the surface(su) moiety) and enabling the entry of the viral nucleoprotein core (mediated primarily by the transmembrane (Tm) moiety). The Su protein binds to its receptor on the target cell surface and, as a result, the Su / Tm complex undergoes a conformational change which catalyzes fusion of the viral and cellular membranes, followed by the entry of the viral core containing the RNA genome into the cytosol of the target cell (figure 2).

The development of retroviral vectors which can be targeted to specific cell types might allow the designing of novel delivery strategies to reduce the infection of non-target cells. The potential for retroviruses to bind and fuse (the infection process) with target cell membranes is solely determined by the interaction of the retroviral envelope proteins (encoded by the *env* gene) with a specific cell surface receptor. As a result of this, the host range specificity is altered by any modification in the *env* gene. The HIV is a classic example of some retroviruses, which naturally possess a restricted target cell population. The specific host cell receptor, the CD4 molecule, is only expressed on a limited number of cell types. There have been attempts to modify the HIV genome to devise safe and efficient HIV-based retroviral vectors. Besides potential toxic activity of the HIV *env* gene product, the existence of a complex regulatory system in the virus has hindered progress in this area (Miller & Vile 1995, Naldini et al. 1996).

Out of a variety of retroviruses potentially exploited for gene transfer, two groups of the Moloney murine leukemia viruses (MoMLV) have so far received much importance. The MoMLV-E group bears the ability to infect virtually all rodent cells (termed ecotropic viruses) while the MoMLV-A group infects a wide variety of mammalian cells including human (termed amphotropic viruses). Some of the most promising protocols (figure 3) used for targeting these particular retroviral vectors are discussed below (Harris & Lemoine 1996).

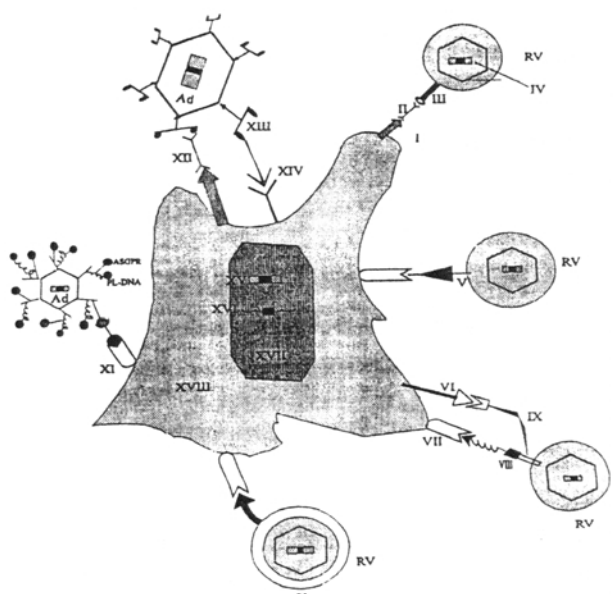

Figure 3 Targeted viral vectors for gene transfer I specific cell surface antigen; II, Bispecific Antibody; III, Envelope protein; IV, therapeutic gene; V, genetically modified envelope; VI, viral receptor; VII, EGF receptor; VIII, EGF; IX, specific protease cleavable linker; X, Pseudotyped virus; XI, Asialoglycoprotein receptor (ASGPR); XII, Bispecific antibody; XIII, Ligand (Hormone, Sugar, Peptide, etc.); XIV, Receptor; XV, Episomal; XVI, Integration; XVII, Nucleus; XVIII, Target Cell; RV, Retrovirous; Ad, Adenovirus; PL-DNA, Polylysine-complexed DNA

Engineering of the retroviral envelope (env) gene: A possibility which has emerged recently is to substitute a portion of the *env* gene with that of another virus to change the tropism of the virus. The *env* gene sequences necessary for correct recognition of the target cell receptors has been identified and can be directly substituted by sequences that encode new non-viral ligands. However, the specific binding of a virus to host cell surface receptors probably involves a complex interaction of several parts of the protein. Therefore, direct modification of a single site in the *env* gene is unlikely to be effective in general. This is further complicated by the fact that, in addition to attachment functions to host cells, the envelope protein also catalyzes fusion-mediated viral entry. Hence, a modification of the virus envelope protein can produce the desired effect of altering the tropism of viral binding but might be detrimental to subsequent steps of viral gene transfer and internalization. Despite these limitations, there have been several attempts of successful modification of the retroviral *env* gene. A 16 amino acid sequence (consisting of arginine-glycine-aspartate: RGD) has been used to modify the avian leukosis virus envelope protein. Such hybrid retroviral particles containing both the modified *env* gene product and the wild type envelope protein were capable of infecting cells expressing integrin that recognizes the RGD motif, albeit inefficiently. A fusion protein consisting of the Rous sarcoma virus signal peptide and influenza virus haemagglutinin was constructed. It was shown that the virions produced from the chimeric envelope protein had a broad host range with the chimeric protein functioning as efficiently as the wild type envelope (Harris & Lemoine 1996). In an attempt to generate a retroviral vector, Kasahara et al. replaced a portion of the envelope protein near the N-terminus with 150 amino acids of the erythropoietin (EPO) protein. Such chimeric virions were able to infect cells carrying EPO-receptors with higher efficiency than cells not expressing the receptor (Kasahara et al. 1994). Somia et al. grafted a single chain variable fragment (scvf) directed against the low density lipoprotein receptor (LDLR), onto the ectotropic envelope of MoMLV. The recombinant MoMLV containing the chimeric envelope was able to infect cells through LDLR recognition (Somia et al. 1995). Another report in this direction has established the potential of a new method of vector targeting in which a single chain antibody directed against a cell surface antigen was fused with an ecotropic MoMLV envelope protein. A related method has been described in which a fusion of the C-terminal portion of the envelope protein of spleen necrosis virus with a single chain antibody was constructed. Retroviral particles bearing the chimeric envelopes were able to bind and infect antigen coated cells but only with an efficiency of approximately 2% compared to that of wild type virus (Harris & Lemoine 1996). It appears that while such engineered ligand domains can produce high affinity binding to selected receptors, there can be a failure to trigger subsequent events required for efficient viral entry. An apparent solution to this problem has been described by Nilson et al. who fused epidermal growth factor(EGF) to an amphotropic retroviral envelope protein via a cleavable linker comprising a factor Xa protease recognition signal. Viral proteins displaying such an envelope attach to cells specifically via the epidermal growth factor receptor whereupon cleavage with the specific protease releases the virus and allows infection through its natural viral receptor Nilson et al. (1996).

Chemical modification of the envelope protein: This approach involves the conjugation of an antibody to the retroviral envelope protein and linkage of this antibody to a second cell-surface-specific antibody using streptavidin. This structure has already shown promising results using antibodies against major histocompatibility complex antigens, EGF, and transferrin receptors expressed on hepatoma cells. However, despite binding and internalization of the modified retrovirus, there was no integration or replication of the provirus. A possible explanation may be that the receptor (and the modified virus bound to it) enters a cellular compartment that does not allow access to the nucleus. The end result is that it is either degraded or recirculated to the cell membrane. Therefore, special consideration will be needed in the selection of target protein for this kind of approach with reference to their potential for access to the nucleus after internalization (Harris & Lemoine 1996).

Another approach was quite successful in modifying the host range of retroviruses through the chemical coupling of lactose to the envelope glycoprotein to allow them to bind specifically to asialoglycoprotein receptors (ASGP-R) found on the surface of hepatocytes. Ecotropic viruses modified in this way could infect human hepatocytes at high efficiency, which is rather surprising, as the asialoglycoprotein endocytic pathway is ultimately a degradative route where the ligands are delivered to lysosomes. One potential explanation for this fact may be that the initial phase of acidification within the endosome favors the conditions for acid-mediated fusion of the viral envelope with the endosomal membrane allowing the virus to escape into other cellular compartments (Harris & Lemoine 1996).

Specificity generation of envelope binding through pseudotyping: The dissection of cis- and trans-acting functions in MoMLV-packaging cell lines allows the easy manipulation of the envelope protein because it may be supplied in trans. The technique known as pseudotyping has enabled substitution of the usual viral envelope with another. Of late, much interest has been documented regarding pseudotyping of retroviral vectors using the G protein of vesicular stomatis virus (VSV). MoMLV-derived retrovirons showed efficient encapsulation of the VSV G protein. The pseudotyped virus offers a number of advantages over the current MoMLV-derived vectors. Primarily, the virions withstand the shearing forces encountered during ultracentrifugation, which allowing them to be concentrated for the generation of a high titer retroviral vector system. Secondly, the viruses can also integrate more than one copy of their genome per host genome and finally can also infect a number of other cell types previously non-infectable by MoMLV-derived vectors. Inspite of these above considerations, the toxicity of VSV G protein to some cell types limits its scaling-up process (Harris & Lemoine 1996).

Adenoviral Vectors

The adenoviral capsid has an icosahedral structure in which the faces are composed of hexon subunits and the vertices consist of penton bases (figure 3). The penton bases contain five copies of the integrin-receptor binding motif RGD, used by the virus to interact with the vitronectin-binding intigrins, $\alpha_v\beta_3$ and $\alpha_v\beta_5$. The interaction of adenovirus with target cells takes place in two stages: (i) the fiber binds to an (as yet uncharacterized) primary receptor on the cell surface, and then (ii) interaction of the penton bases with the integrin receptors triggers the internalization. The major problem in this kind of interaction is the fact that most cells possess the primary receptor for the adenovirus fiber protein (Harris & Lemoine 1996). There are evidences that the fiber can be modified to allow the coupling of other molecules in order to redirect binding specificity. Cristiano et al. have reported covalent conjugation of asialo-orosomucoid-poly (L-lysine) to adenovirus particle so as to deliver a DNA vector containing canine factor IX to mouse hepatocytes (Cristiano et al. 1993). The results suggest that receptor-mediated endocytosis coupled with the property of efficient endosomal lysis induced by adenovirus should permit the application of targeted and efficient gene delivery systems for the liver. In a similar attempt, Wu et al. have modified adenovirus type 5(Ad5) by coupling an asialoglycoprotein-polylysine conjugate to the virus by reactions that activate carbohydrate residues exclusively located on fibers (Wu et al. 1994). Using a β-galactosidase marker gene, the number of liver cells bearing (ASGP-R) transfected by modified virus was found to be 200-fold higher than the asialoglycoprotein-polylysine DNA complex alone. This strategy took advantage of the fact that the adenovirus contains carbohydrate groups that are exclusively located on the fibers. Furthermore, Takahashi et al. have shown which after systemic administration to Gunn rats, recombinant adenovirus type 5 expressing human bilirubin-UDP-glucuronosyltransferase-1 (BUGT1) localizes preferentially in the liver and can express the transgene in nearly 90% of the hepatocytes. As a result of this treatment, these Gunn rats deficient in BUGT1 resulted in biliary excretion of bilirubin glucuronides and showed 70% reduction of serum bilirubin levels. However, the effect was transient and host humoral and cellular response prevented transgene expression

after subsequent injection (Takahashi et al. 1996). Wickham et al. (1996) have demonstrated that the bispecific antibody approach can be successfully exploited to target adenoviruses to a specific cellular receptor and thereby increase the efficiency of gene transfer (Wickham et al. 1996). Using a similar approach, adenoviruses can be targeted to T cells via non-adenoviral receptors resulting in efficient transduction of the foreign gene. (Wickham et al. 1997).

Targeted Molecular Conjugates

It is known that polylysine can be complexed with DNA via a polycation. Several molecular conjugates have been devised including asialoglycoprotein ligand conjugates to target hepatocytes and IgA-ligand conjugates in targeting respiratory epithelial cells. The major hurdle involved with these types of delivery vehicles is that upon entry they are mostly localized mainly in the endosome, thereby limiting the efficacy of gene transfer (Harris & Lemoine 1996). To circumvent these problems, adenovirus has been included in the conjugated vectors because of their endosomolytic activity. Inspite of their efficient entry into cells *in vitro*, their use *in vivo* has been limited due to the instability and site constraints (Wu et al. 1994).

Inducible Promoters in Cell Type Specific Gene Delivery

The important issue in clinical application of gene therapy is the dire need for increased gene transfer efficiency and target specificity involved with controlled gene expression at therapeutically relevant levels *in vivo*. Elements regulating gene expression, such as promoters and enhancers, possess cell type specific activity and can be activated by certain induction factors (e.g., hormones, cytokines, cytostalins, heat shock, irradiation, etc.) via responsive elements. Using such regulatory elements as internal promoters to derive the expression of therapeutic genes in viral vector constructs, a controlled and restricted expression of these genes can be achieved (figure 4). Besides high level and efficient gene expression, minimizing or excluding inappropriate gene expression in the surrounding non-target cells is of utmost importance for the design of various gene therapy protocols. Some of the promoters that represent attractive candidates for viral vector construction are listed in table 1.

Among viral and non-viral delivery systems, retroviral vector-mediated gene transfer is the most extensively studied gene transfer system due to high transfer efficiency in conjunction with stable integration into the host genome. However, it is

Table 1 *Various cell type specific promoters for targeted gene expression*

Promoter	Target Cell/Tissue	Gene
Immunoglobulin Promoters	B-Lymphoma	Diphtheria toxin A
β-Globin promoter	Erythroid cells	β-Globin
Phosphoenolpyruvate Carboxykinase (PEPCK)	Hepatocytes	Growth hormone gene, Clotting factor IX
Albumin Promoter	- do -	Alpha I antitrypsin
Tyrosinase Promoter	Melanomas	β-galactosidase
α-fetoprotein promoter	Hepatocellular Carcinomas	Varicella zoster virus Thymidine kinase
Human surfactant Protein C	Broncheolar and Alveolar epithelium	Cystic fibrosis transmembrane Conductance regulator

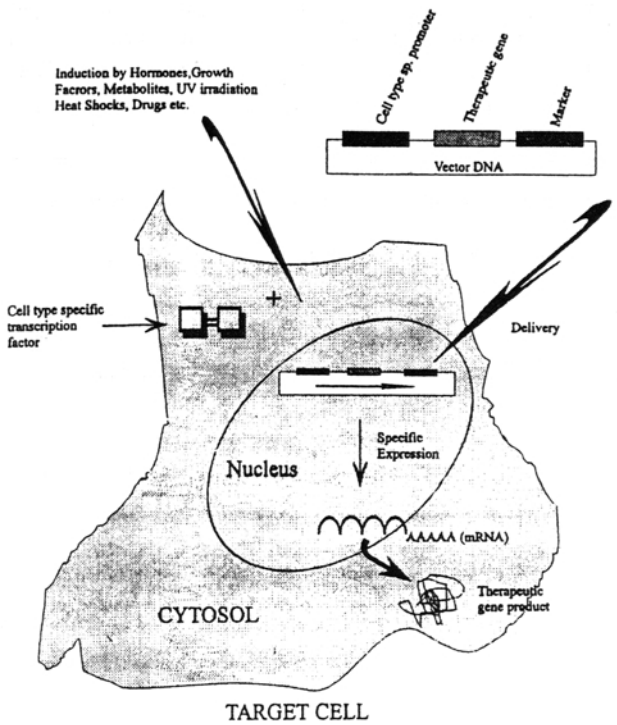

Figure 4 Promoter controlled gene targeting

noted that some viral promoters are inactivated after a certain time in the transduced cells, affecting foreign gene expression. Therefore, cell-type specific promoters, as available for many tissues, could be used as internal promoters in viral and other targeted non-viral vector constructs for foreign gene expression (Walther & Stein 1996).

Liposome Mediated Targeted Gene Transfer

The vector system plays a pivotal role in expressing a foreign gene in the target cells to ensure it must not only target the appropriate cell type but also allow efficient delivery of undegraded DNA to the nucleus. In a number of clinical trials, liposomes have already been used to deliver DNA into a variety of cell types (Harris & Lemoine 1996). The use of conventional liposomes is limited for this purpose, first because of their specific uptake by cells of the reticuloendothelial system (RES), in particular by macrophages resident in liver, spleen, bone marrow, etc.; and second because of their internalization into target cell lysosomes through receptor-mediated endocytosis. Although RES uptake could be controlled by the use of "stealth" liposome formulation, this route of entry causes an extensive degradation of delivered DNA mediated by lysosomal enzymes. Conjugation of liposomes with antibody or ligands has developed ways for targeting liposomes to selective cell types. However, it is not sufficient to confer on such liposomes a particular binding ability but to have the ability of the ligands to allow fusion of liposomes with cell membrane. This is expected to deliver the liposomal content (DNA) directly to the cellular cytosol, by passing the lysosomal degradative pathway in case of receptor-mediated entry. This kind of cytosolic delivery of DNA is especially important for gene-delivery vectors, where the DNA must not only reach the appropriate cell type but must also reach the nucleus in intact form. Sendai virus or its fusion glycoprotein (F) has been used with various liposomal formulations to promote cytosolic delivery of aqueous contents (protein, drugs, DNA, etc.) to various cell types in culture (Bagai & Sarkar 1993, 1994, Ramani et al. 1997). Further developments in this area may finally lead to a "super-liposome" having all of the above characteristics to be used both *in vitro* and *in vivo*

Development of a Novel Gene Delivery Vehicle To Liver Cells Using Engineered Sendai Viral Envelope

A major problem in the delivery of DNA and other biological macromolecules into cells is the crossing of the permeability barrier imposed by the plasma membrane. Various methods have been employed to generate effective gene delivery vehicles needed for therapeutic application. In the past few years, various delivery vehicles like adenoviral vectors, retroviral vectors, and non-viral vectors like liposomes and DNA-polylysine complexes have been extensively used for the transfer and expression of reporter genes as well as therapeutic genes both *in vitro* and *in vivo* (Harris & Lemoine 1996). These methods have been modified to generate high efficiency vehicles, but they suffer from many other drawbacks which limit their use for *in vivo* application. In spite of all recent developments in gene therapy since 1989, the formulation of a targeted gene delivery "vector" is still far from ideal.

Reconstituted Sendai viral envelopes (F, HN-virosomes) containing two glycoproteins, F (fusion protein) and HN (hemagglutinin-neuraminidase) are known to fuse efficiently with the plasma membrane of target cells and are excellent carriers for fusion-mediated microinjection of biologically active macromolecules *in vitro* (Bagai & Sarkar 1994). This delivery system utilizes the binding of HN to the sialic acid residues of the membrane, followed by the F-protein-mediated fusion of the viral envelopes with the host cell plasma membrane at neutral *p*H. However, this promising system of gene delivery lacks cell-type specificity because of the presence of HN protein known to bind to various cell types through the sialic acid moiety of cell surface glycoconjugates. It has been recently demonstrated in our laboratory that F-virosomes (devoid of HN protein) can specifically bind and fuse with HepG2 cells (Bagai & Sarkar 1994). The target specificity of F-virosomes has been ensured by the strong interaction between the terminal β-galactose moiety of F protein and the asialoglycoprotein receptor (ASGP-R) on the membrane of HepG2 cells. In further studies, F-virosomes have been successfully used for the delivery of biologically active macromolecules into the cytoplasm of liver cells both *in vitro* and *in vivo* (Bagai & Sarkar 1993, 1994, Ramani et al. 1997). Liver is known to be a model organ for somatic gene therapy. Hence, F-virosomes by virtue of their specific interaction and fusion with liver cells should be an ideal vector for gene delivery both *in vitro* and *in vivo*. We have reported for the first time the F-virosome-mediated delivery of CAT gene and its expression in HepG2 cells in a systematic and quantitative fashion (Ramani et al. 1997). Our findings of the specific interaction of Sendai viral fusion glycoprotein (F) with the human asialoglycoprotein receptor (ASGP-R) is of considerable importance for the development of safe and efficient hepatotropic vectors coveted for *in vivo* liver gene therapy applications. Based on interaction with ASGP-R, viral and non-viral hepatotropic gene transfer systems have been employed both *in vitro* and *in vivo*. While partial targeting to hepatocytes was achieved, the efficiency of these vectors was found to be severely impeded because of the lysosomal degradation of endocytosed ligands (Wu et al. 1994). Interestingly, in case of F-virosomes, the F-protein acts in a bifunctional way; i.e., binding to hepatocytes followed by membrane fusion-mediated direct release of the virosomal aqueous contents to the cytoplasm of target cells (Bagai & Sarkar 1994). Having established F-virosome-mediated efficient delivery of foreign genes specifically into human hepatoblastoma cells in culture, we assessed the ability of DNA-loaded F-virosomes to affect targeted gene expression in mice following tail vein injection.

Protocols

Construction and isolation of eukaryotic expression vectors: pCIS3CAT; A 1.55-kb Xho1-Sma1 fragment from plasmid pTKCAT containing CAT gene and SV40 polyadenylation signal was cloned into the plasmid pCIS2, downstream of the cytomegalovirus promoter-enhancer element. Ligation mixture was used to transform *E.coli* DH5α following a standard protocol (Sambrook et al. 1989). A putative clone was identified and confirmed by restriction mapping and Southern hybridization using ^{32}P-labelled CAT gene fragment as a probe. This putative clone was designated as pCIS3CAT. Plasmid, **pBVluc** (6.38 Kb), containing the firefly luciferase gene under control of CMV promoter, was derived from pCEP4-X2 luc (Stratagene, La Jolla, CA). In pBVluc, the luciferase coding sequence is followed by SV40 polyadenylation sequence. These plasmids were isolated and purified using Qiagen Megaprep columns. DNA concentration was determined by measuring the absorbance at 260nm.

Preparation of F-virosomes loaded with pCIS3CAT and pBVluc DNA: Reconstituted Sendai viral envelopes containing the F protein (F-virosomes) were prepared following the procedure standardized in our laboratory (Bagai et al.1993). A suspension of 20mg of Sendai virus (Z strain, grown in ten days old embryonated chicken eggs) in PBS was centrifuged, and the pellet obtained was resuspended in 4ml of buffer A (150mM NaCl, 20mM Tris [*p*H 8.4]) containing 3mM DTT. The suspension was incubated at 37°C for 2 hr and then

dialyzed at 4 to 10°C for 16 hr against 3 changes of 2 liters of buffer B (150mM NaCl, 10mM Tris [*p*H 7.4], 2 mM Ca^{2+}, 2mM Mg^{2+}). The viral particles were centrifuged, and the pellet obtained was resuspended in 2ml of buffer B containing 40mg of TX-100. After incubation at 20°C for 1 hr, the suspension obtained was centrifuged (100,000 × g for 1 hr at 4°C) to remove the detergent-insoluble substances which presumably contain reduced HN and nuleocapsid. From the clear supernatant, the detergent was removed by stepwise addition of SM2 Bio-Beads. Briefly, 320 mg of methanol-washed SM2 Bio-beads was added to 2ml of supernatant and incubated at 4°C with gentle rocking. After 2 hr, an additional 320mg of SM2 Bio-Beads was added, and incubation continued at 20°C for 2 hr. Incubation was further continued for 2 hours at 20°C with an additional 640mg of SM2 Bio-Beads. The turbid suspension was separated from the Bio-beads with a 26-gauge needle and centrifuged as described above. The pellet containing F-virosomes was resuspended (0.5 to 1mg of protein) in 1ml of buffer B and stored at 4°C. For preparation of F-virosomes containing DNA, the Triton X-100 solubilized fraction of virus was mixed with plasmid DNA (75μg of DNA per mg of viral protein) and reconstituted as described above (figure 5). The unentrapped DNA adsorbed on the outer surface of the virosomal membrane was removed by treatment of virosomes with DNAase I (60 μg DNAase I per mg of F protein) at 37°C for 30 min. The presence of entrapped DNA was checked by lysing virosomes with 2% SDS, loading on 0.8% agarose gel, and subsequently staining with ethidium bromide. The amount of DNA entrapped in F-virosomes was calculated using ^{32}P-labeled plasmid DNA as a tracer. Membrane fusion activity of loaded F-virosomes was ascertained by their ability to bring about lysis of mouse RBCs in the presence of WGA(Bagai et al.1993). Structural

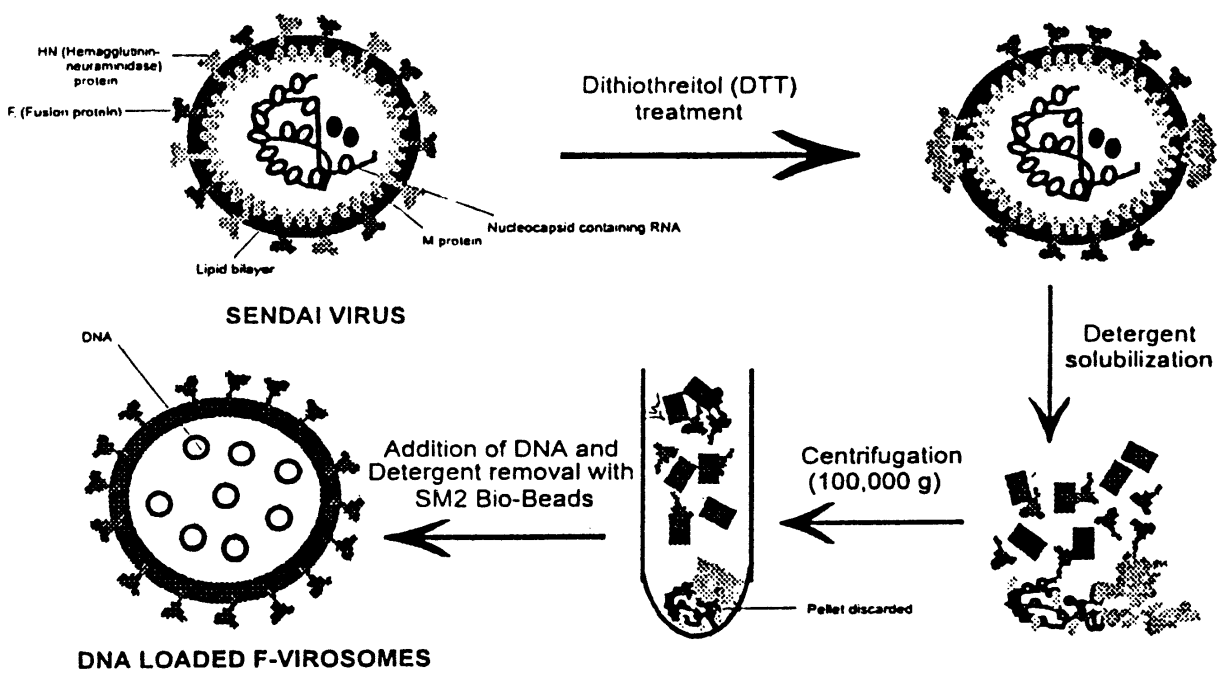

Figure 5 Preparation of DNA-loaded F-virosomes. Treatment of sendai virus with DTT followed by dialysis to remove DTT leads to the formation of large aggregates of several HN proteins. Such reduced Sendai virus is then solubilized with non-ionic detergents like Triton X-100 followed by centrifugation at 100,000 g. The pellet contains detergent-insoluble nucleocapsids, the M protein, and the HN complexes, while the supernatant consists of F-protein along with the membrane lipids. The above supernatant is mixed with DNA and the detergent is removed by stepwise addition of SM2 Bio-Beads leading to the formation of vesicles with only the fusion protein in their membrane along with DNA entrapped in the aqueous phase (DNA-loaded F-virosomes)

integrity of loaded F-virosomes was checked by leakage of entrapped DNA during incubation with phosphate-buffered saline (PBS, 150mM NaCl, 10mM phosphate, *p*H 7.4), with fresh mouse plasma, with 10% FCS containing DMEM at 37°C for 16 hr and after heat-treatment at 56°C for 30min.

Administration of loaded F-virosomes and gene expression in vivo: Twelve week-old female Balb/c mice (~18g) were injected intravenously into the tail vein with DNA-loaded F-virosomes (0.4 mg F protein containing 2μg of DNA) in a final volume of 0.2 ml Tris-buffered saline (Miller & Vile 1995) containing 2 mM Ca^{+2}. Antibody response against F protein was checked as described earlier (Nilson et al. 1996). (a) *RT-PCR amplification of CAT-specific transcripts*: 100mg of total RNA isolated (Cristiano et al. 1993) from liver was incubated with 10 units of DNAase I (Sigma) at 37°C for 15 min. and 1mg of this RNA was reverse-transcribed with 200 units of MMLV-reverse transcriptase (Gibco-BRL, USA). CAT-specific transcripts were amplified by 35 cycles of RT-PCR (1 min at 94°C, 1 minute at 60°C, 1 minute at 72°C and final extension, 1 minute at 72°C) using the antisense primer 5¢ TTA CGC CCC GCC CTG CCA 3¢ from the 3¢ end of CAT and the sense strand primer, 5¢ ACC GTT GAT ATA TCC CAA TGG 3¢ from a sequence 27 nucleotides downstream of the 5¢ end of CAT. The RT-PCR products were electrophoresed on 1.2% agarose gel and transferred to nylon membrane. A 1.5 Kb *Xho* I/ *Sma* I CAT gene fragment derived from the plasmid pCIS3CAT was labeled with α-^{32}P dCTP using random primer labeling technique. The above blots were subsequently hybridized with this probe; and (b) *Detection of CAT protein by ELISA:* Subcellular fractionation of various organs was carried out as described earlier(Bagai & Sarkar 1994). Briefly, the organs were placed in isotonic homogenizing buffer (0.01 M *Tris*-HCl (*p*H 7.4) containing 0.25 M sucrose) and then dispersed in Potter Elvehjem type homogenizer at 4°C. The cytosolic fraction was purified after differential centrifugation of the homogenate. The amount of CAT protein expressed in the cytosolic fraction was quantitated using the CAT ELISA kit (Boehringer Mannheim, Germany).

Detection of CAT gene in liver parenchymal cells by PCR: Parenchymal cell (hepatocyte) separation was carried out following a standard procedure (Celis 1994) (21). To summarize, the perfused liver was excised from the animal, cut into small pieces and washed thoroughly. The liver pieces were incubated at 37°C for 15 min in Collagenase A (Boehringer Mannheim, Germany) containing buffer. The resulting liver mass was filtered through a nylon mesh, and the filtrate was centrifuged at 400 rpm for 10 minutes at 4°C to obtain the pellet containing hepatocytes. The supernatant containing non-hepatocytes was treated with 0.2% pronase E (Sigma, St. Louis, USA) to digest the contaminating hepatocytes completely (Soriano et al. 1983). The non-hepatocyte pellet was obtained by centrifuging the pronase-treated supernatant at 1300 rpm for 10 min at 4°C. Both these cell types were washed three times with ice-cold phosphate-buffered saline containing 5 mM EDTA, and the cell pellets were processed for subcellular fractionation as described above. One microgram of total DNA isolated from each subcellular fraction was subjected to PCR using 1.25 units of AmpliTaq® DNA polymerase (Perkin Elmer-Cetus, USA). A 633 bp fragment of CAT gene was amplified by 35 cycles of PCR (using the conditions mentioned above). The PCR products were visualized on a 1.2% agarose gel.

Measurement of luciferase activity in parenchymal and non-parenchymal liver cells 24 hours post-injection of pBVluc loaded F-virosomes: Following separation, these cells were washed twice with Tricine-buffered saline and suspended in the same. For luciferase assay, the Triton X-100 solubilized cell lysate (from $1x10^7$ cells) was mixed with luciferase assay buffer containing 1mM luciferin (Boehringer Mannheim). Luciferase activity (in mV) was measured in a 1250 Luminometer (BIO-Orbit, Finland).

State of targeted DNA associated with persistent gene expression: Total DNA was extracted from parenchymal cells at 1, 15, 30, and 60 days post-injection. The chromosomal DNA was separated from episomal DNA by gel filtration using Sephacryl S-1000 beads. Both the DNA fractions were ethanol precipitated and finally dissolved in

TE buffer. The chromosomal DNA was made free of any contaminating episomal DNA by electrophoresis on a 1% low melting point agarose (LMP) gel. Following electrophoresis of the chromosomal DNA (undigested) on 0.7% agarose gel, the blots were hybridized with 1.5 Kb-CAT gene fragment. Purified chromosomal DNA digested with various restriction enzymes (New England Biolabs, Inc. USA) was hybridized as described above. The chromosomal and episomal fractions were analyzed by PCR using CAT-specific primers. The PCR cycles were as follows: initial denaturation, 94°C for 10 min; denaturation, 94°C for 1 min; annealing, 60°C for 1 min; extension, 72°C for 1 min; final extension, 72°C for 10 min. After 35 cycles, the products were electrophoresed on 1.2% agarose gel, transferred to nylon membrane, and the blot was hybridized with 1.5 Kb-CAT gene fragment.

Results

Characterization of DNA-loaded F-virosomes: F-virosome preparations were examined for purity by SDS-PAGE in the presence of β-mercaptoethanol (Bagai et al.1993) and were found to be free from any detectable contamination of other proteins. Membrane fusion activity of these virosomal preparations was checked by their ability to lyse mouse RBCs in the presence of WGA. F-virosome associated plasmid DNA was DNAase I resistant, indicating it was entrapped rather than adsorbed on the virosomal membrane. No detectable leakage of DNA was observed from F-virosomes incubated with PBS or plasma/FCS and heat-treated F-virosomes. Two to five microgram of intact DNA was found to be encapsulated in 1mg of F-virosomes.

CAT gene expression in vivo after administration of DNA-loaded F-virosomes: The CAT gene expression was assessed both at the level of mRNA and of protein. Maximum CAT expression was achieved in the liver at a dose of 2μg of DNA loaded in F-virosomes . RT-PCR analysis of total RNA from various organs confirmed the presence of CAT-specific transcripts in mouse liver (figure 6). Other organs analyzed (lung, kidney, and spleen) did not express significant CAT transcripts except a very faint RT-PCR signal in the case of lungs. CAT gene expression was not detected in tissues like heart,

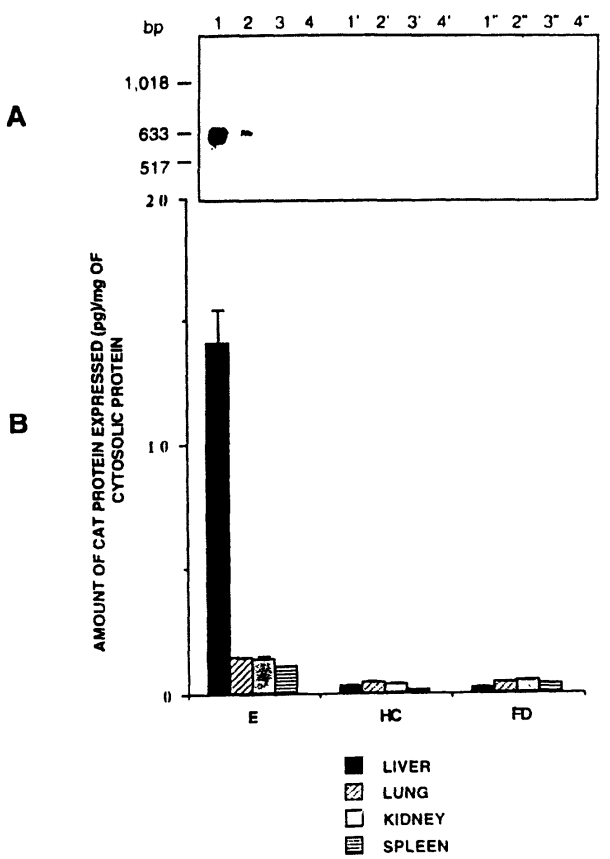

Figure 6 Tissue-specific expression of CAT gene 24 hours post-injection. (A) RT-PCR amplification of CAT mRNA from various organs using CAT-specific primers followed by hybridization with 1.5 Kb labeled CAT gene probe. Lanes 1,2,3,4, RT-PCR products from liver, lung, kidney, and spleen, respectively; Lanes 1¢-4¢, corresponding samples from mice injected with heat-treated loaded F-virosomes; Lanes 1¢¢-4¢¢, corresponding samples Each point represents mean value (± SD) from three animals. E, experimental; HC, heat control; FD, free DNA

muscle, brain, lymph nodes, skin, and tail. Heat-treated virosomes and the free DNA controls also did not exhibit any CAT-specific transcripts. The relative expression of CAT protein in the liver was comparable to mRNA levels using the same category of F-virosomes (figure 7). The expression of both mRNA and protein in mouse liver persisted over a period of 60 days (figure 7), pointing to the overall efficiency of this delivery system. The expression was also detected until 120 days (figure 8) with no F-specific humoral antibody response from ten days onwards. Moreover, the animals remained healthy and active during the experiments.

Fusion-mediated uptake of pCIS3CAT DNA by liver parenchymal cells: Experiments were designed to determine the cellular localization of the input DNA. Total DNA from various subcellular fractions of these cells was analyzed by PCR using CAT-specific primers which amplify a 633 bp fragment of CAT gene. After 2 hr following injection of loaded F-virosomes, plasmid DNA was detected only in the cytosolic fraction (figure 9). After 6 hr, the majority of the DNA was localized in the nucleus and was detected mainly in this compartment till 24 hr. No DNA was detected in the lysosomal/mitochondrial fraction at any time post-injection. In case of the corresponding heat-treated virosomes, the delivered DNA persisted mainly in the lysosomal/mitochondrial fraction till 12 hr and finally could not be detected in any fraction after 24 hr. No PCR amplified product was detected from any subcellular fraction of non-parenchymal cells or following injection of free DNA.

Cell-type-specific luciferase gene expression in mouse liver: The luciferase activity was found to be 8 to 10 times greater in parenchymal cells compared to the non-parenchymal counterparts. Moreover, the luciferase expression in parenchymal cells from experimental mice was 3 to 4 times more than that of the corresponding heat controls. In case of non-parenchymal cells, experimental and heat control exhibited little difference in luciferase activity (figure 10). The purity of the cell preparations was monitored by pronase susceptibility and glass slide attachment of the non-parenchymal cells.

Integration of the delivered DNA in the chromosome of hepatocytes: To determine the state of targeted DNA in hepatocytes after administration of loaded F-virosomes, purified chromosomal DNA was hybridized to labeled 1.5 Kb CAT gene fragment derived from pCIS3CAT. No hybridization signal was detected from purified chromosomal DNA one day post-injection; however, chromosomal DNAs from hepatocytes 15, 30, and 60 days post-injection contained high molecular weight sequences that hybridized with the CAT gene probe (figure 11). Chromosomal DNA from mock-injected mice, Herring sperm DNA (Gibco-BRL, USA), and DNA from non-parenchymal cells failed to show any hybridization signal. To establish the nature of integration of the DNA in mouse chromosome,

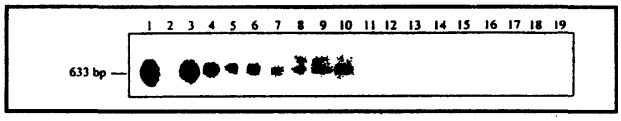

Figure 7 RT-PCR Amplification of cat Transcripts at various time intervals post-injection. RT-PCR amplification of CAT transcripts at various time intervals post-injection. The different lanes are: lane 1, 633 bp CAT gene fragment amplified from pCIS3CAT; lanes 2 to10, RT-PCR products from experimental mice at 0.25, 1, 2, 4, 6, 8, 15, 30, and 60 days post-injection; lanes 11 to 19, the corresponding heat controls

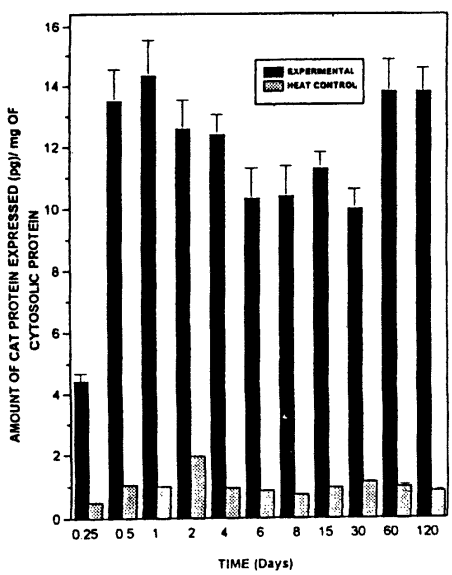

Figure 8 Time-dependent expression of CAT protein in mouse liver. CAT expression was quantitated in cytosolic fractions of liver cells by CAT ELISA. Each value represents mean (± SD) from three animals

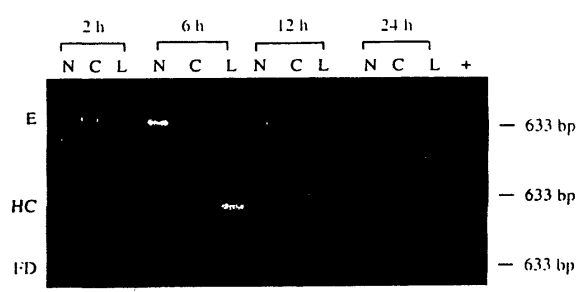

Figure 9 PCR amplification of the delivered DNA in various subcellular fractions of hepatocytes. Total DNA from various subcellular fractions of parenchymal cells was analyzed by PCR using CAT primers. The panels on the left are E, experimental; HC, heat control; FD, free DNA. The different lanes are N, nuclear; C, cytosolic; L, lysosomal/mitochondrial. The 633 bp CAT gene fragment amplified from pCIS3CAT was used as a control (+)

purified genomic DNA from mouse liver 60 days post-injection, was digested with various sets of restriction enzymes and the resulting fragments were analyzed after hybridization with 1.5 Kb CAT gene probe. Purified chromosomal DNA, isolated from CAT injected animals and restricted with different sets of enzymes, generated CAT-positive fragments of lower size than that of the linearized plasmid (6.7 Kb) and a few hybridizable fragments greater in size than the linearized plasmid (figure 11). Since these band patterns do not correspond to those obtained after digestion of pCIS3CAT, this suggests the possibility of random integration of the delivered plasmid in the mouse chromosome. The integration status was further confirmed by PCR analysis of the purified chromosomal and episomal fractions using CAT-specific primers. Twenty four hours post-injection, amplified CAT gene fragment (633 bp) was detected only from the episomal fraction, but no such product was obtained from the corresponding chromosomal

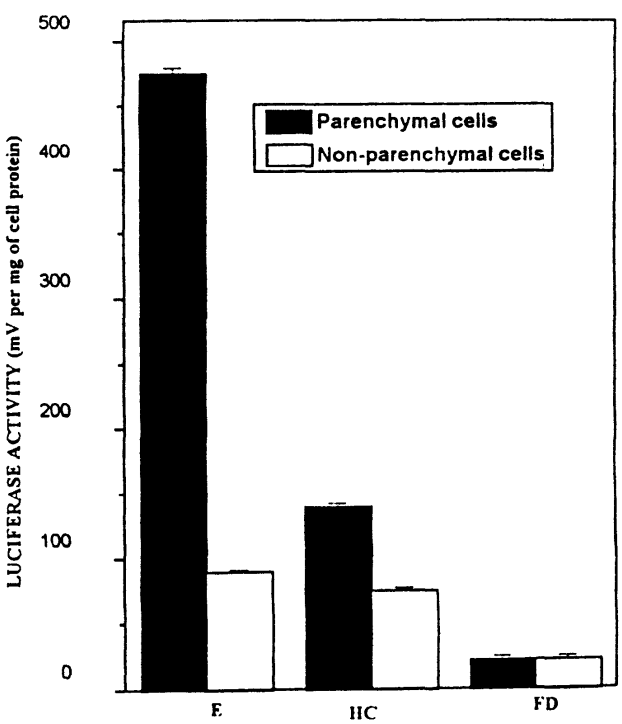

Figure 10 Preferential expression of Luciferase gene in parenchymal cell types of mouse liver. Luciferase activity in parenchymal and non-parenchymal cell types was determined as described in "Experimental protocol." Values represent mean (± SD) from four mouse livers. E, experimental; HC, heat control; FD, free DNA.

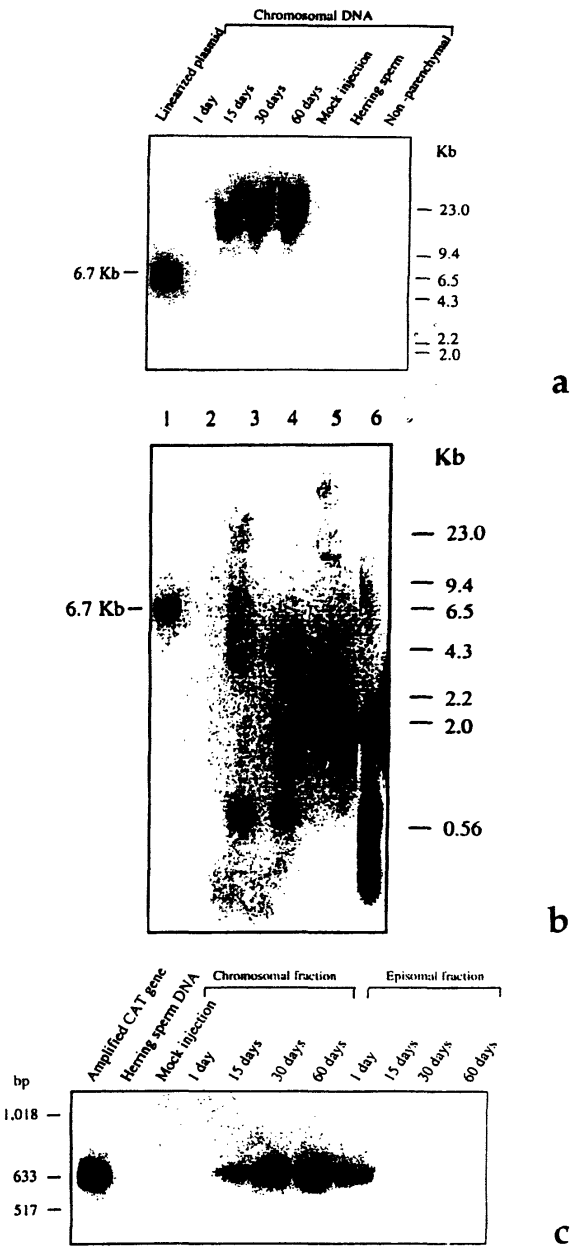

Figure 11 Integration status of CAT gene in liver parenchymal cells. At different time points post-injection, total DNA was isolated from hepatocyte nuclei, and the chromosomal pool was purified from the low molecular weight episomal fraction as described in "Experimental protocol". (**a**) Southern hybridization of undigested chromosomal DNA with labeled CAT gene probe; (**b**) Southern hybridization pattern of chromosomal DNA (from a single mouse) digested with various restriction enzymes. Lane 1, Linearized pCIS3CAT-*Xho* I digest; Lane 2, Genomic DNA from mock injected animals after *Eco*R I digestion; Lanes 3, 4, 5, and 6, Genomic DNA from experimental animals 60 days post-injection after digestion with *Eco*R I, *Pst* I/*Pvu* II and *Alu* I/*Hind* III and *Xho* I respectively; (**c**), PCR of purified chromosomal and episomal fractions from liver nuclei using CAT primers

fractions. On the other hand, 15, 30, and 60 days post-injection, amplified products were detected only from the chromosomal fractions but not from the corresponding episomal fractions. The possibility of non-specific PCR amplification was ruled out from appropriate negative controls (figure 11C). The copy number of the plasmid integrated in the genome was found to range from about 100 to 300 copies per cell as calculated from slot blot hybridization of chromosomal DNA from hepatocytes 60 days post-injection.

Notes

The present findings constitute a rational and quantitative approach to targeted delivery and expression of a foreign reporter gene (CAT) in liver cells using a novel vehicle derived from Sendai viral envelopes (F-virosomes) with the added novelty of avoiding the degradation of the entrapped DNA caused by the endocytotic pathway. The target specificity of F-virosomes has been ensured by the strong interaction between terminal β-galactose moiety of F-protein and asialoglycoprotein receptor (ASGP-R) on the membrane of HepG2 cells (Bagai et al.1993). We have recently established in our laboratory that F-protein behaves both as a ligand and membrane fusogen for targeting of virosomal aqueous contents to the cytosolic compartment of liver cells both *in vitro* and *in vivo* (Bagai & Sarkar 1993, 1994, Ramani et al. 1997). It was shown previously that heat-treatment of F protein in F-virosomes completely abrogates its fusogenic potential without significantly affecting the galactose-mediated specific recognition of ASGP-R (Bagai & Sarkar 1994). This has suggested the use of heat-treated F-virosomes loaded with DNA, as liganded proteo-liposomes which may be efficiently endocytosed by HepG2 cells. The art of natural ligand-receptor interaction and Sendai viral F glycoprotein-catalyzed membrane fusion has been exploited in the construction of a safe and efficient vehicle (F-virosome) for site-specific gene delivery to mouse liver cells *in vivo*. The F glycoprotein is known to possess Le^x(Galβ1-4 [Fucα1-3]GlcNAc-) terminated biantennary oligosaccharides (Yoshima et al. 1981). The Le^x moiety possesses much higher affinity for ASGP-R present on hepatocytes. This property is considered to be a prerequisite of targeted delivery systems. The liver is an ideal organ for transfection of a gene whose product is secreted into the circulation and is important for systemic gene therapy for several inherited diseases. Moreover, the nonpathogenicity of Sendai virus to humans and its little immunogenicity have together increased the prospects of F-virosomes in gene therapy (Tomita et al. 1996). The results presented here using two reporter genes independently are highly supportive of this notion.

It has been shown earlier that heat-treated loaded F-virosomes deliver genes to the lysosomal compartment of the cell following the receptor-mediated route of entry (Ramani et al. 1997). The higher efficiency of fusion-mediated delivery over receptor-mediated entry is very much pronounced below 3μg DNA dose. The hepatotropic nature of this gene carrier is clearly revealed both in terms of mRNA and protein levels. More striking is the sustained nature of gene expression. These features represent a novel instance of targeted and stable gene expression *in vivo* with no detectable adverse immune effects. Recently, attempts have been made to use liganded cationic-liposomes for transgene expression *in vivo*. Besides the known risk of toxicity of cationic lipids and the transient expression of CAT gene, the efficiency of this system is about 10 to 12 times less (Templeton 1997) than that of the F-virosomal delivery. In spite of a sustained insulin gene expression through Sendai virus-liposome complex, this system lacks cell-type specificity and may have undesirable side effects because of the presence of the sialic acid binding protein (HN) of viral origin (Tomita et al. 1996). The "Trojan Horse" strategy of reconstituted Sendai virus envelope in the field of drug delivery and gene therapy as reviewed earlier (Blumenthal & Loyter 1991) also suffers from the lack of target specificity. Moreover, attempts have been made to deliver preproinsulin I gene through lactosylceramide containing liposomes *in vivo*. These vesicles are known to be internalized through ASGP-R on liver cells (Soriano et al. 1983). The transient expression (until four hours) resulting from these experiments corroborates with the endocytotic uptake of these loaded liposomes (leading to the lysosomal degradation of the

transgene) in contrast to the fusion-mediated delivery through F-virosomes. Besides this, the known Le^x type of ligand present on F-virosomes confers it with the added novelty of selective targeting to specific cell types *in vivo* over the existing modes of DNA targeting.

The F-virosome mediated targeted cytosolic gene delivery *in vivo* to liver parenchymal cells is apprehended from the cytosolic localization of plasmid DNA at 2 hr post-injection and from its absolute nuclear localization 6 hr post-injection onward. This is additionally substantiated from the lysosomal localization of plasmid when delivered through heat-treated F-virosomes (Ramani et al. 1997). The overall efficiency of this delivery mode is also reflected from the absence of any detectable PCR signal upon intravenous administration of free DNA. This is probably the first systematic demonstration of intracellular trafficking of a foreign gene administered *in vivo*. Another strong evidence of this membrane fusion-mediated targeted gene delivery is the preferential expression of luciferase gene in the parenchymal cell-types of mouse liver.

The persistent nature of transgene expression in hepatocytes may be explained from the stable integration of CAT gene in the mouse chromosome. It is pertinent to note that sustained gene expression does not appear to be affected by the random nature of integration of pCIS3CAT DNA. The F-virosomes, being at the interface of viral and non-viral vectors, are adequately safe in comparison to their non-hybrid counterparts (Verma & Somia 1997). These attributes altogether highlight the potential of this gene carrier for targeted delivery of therapeutic genes both *in vitro* and *in vivo* (Tsuboniwa et al. 2001, Nijhara et al. 2001a, Nijhara et al 2001b).

Future of Gene Therapy

The real challenge in the field of gene therapy for human diseases lies in translating many vibrant ideas into reality, which could be finally applied in the clinic. The major hurdle is restricted to the poor efficiency of "gene delivery process" *in vivo* with the presently available technology. The properties of both viral and synthetic systems in conjunction may constitute an ideal gene courier. These are as outlined as below:

- •High titre viral particles allowing many cells to be infected.
- •Ease and reproducibility of production.
- •Targeting of the desired cell type.
- •Possession of a transcriptional unit capable of manipulation by regulatory elements.
- •Site-specific integration of the transgene in the host chromosome or of its maintenance as a stable episome.
- •Components should be non-immunogenic.

No such kind of vector is available so far. However, we do hope this will be overcome by further engineering of viral vectors and synthetic systems and by combining the best elements of these carriers (figure 12).The focus will be to

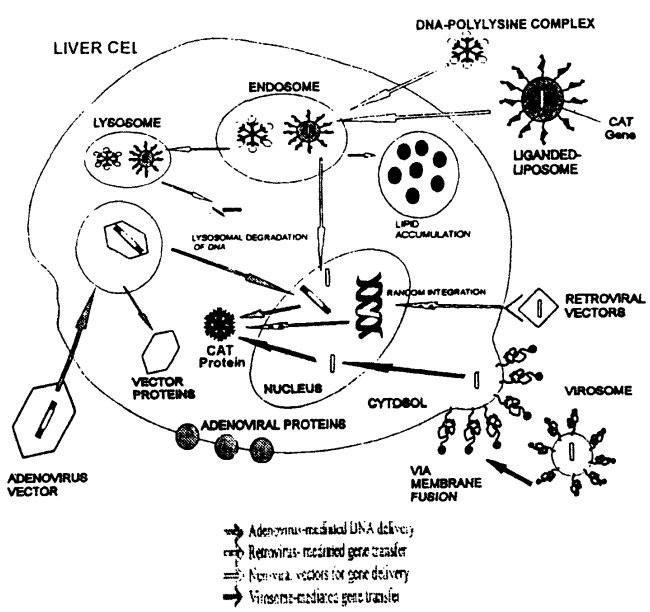

Figure 12 Outline of Various modes of gene delivery using CAT as an example

understand the mechanism controlling of gene expression in target cells so as to approach long-term therapy. With the advent of advanced information on gene promoters and enhancers combined with our understanding of chromatin structure and function emanating from the "human genome project," we should eventually design and control the coveted vector for lifetime use.

Acknowledgements

We are delighted to dedicate this article in the fond memory of our beloved **"Guruji"**, the late Prof. Bimal K Bachhawat. We are also thankful to the Department of Biotechnology, Council of Scientific and Industrial Research, University Grants Commission, Govt. of India, for financial assistance.

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