Genomic Context Vectors and Artificial Chromosomes for Cystic Fibrosis Gene Therapy

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Abstract: Cystic fibrosis (CF) is caused by mutations of the CF transmembrane conductance regulator (CFTR) gene, which encodes a cAMP dependent chloride channel whose expression is finely tuned in space and time. Gene therapy approaches to CF lung disease have demonstrated partial efficacy and short-lived CFTR expression in the airways. Drawbacks in the use of classical gene transfer vectors include immune response to viral proteins or to unmethylated CpG motifs contained in bacterially-derived vector DNA, and shut-off of viral promoters.

These limitations could be overcome by providing stable maintenance and expression of the CFTR gene inside the defective cells. This strategy makes use of large fragments of DNA of various sizes containing the CFTR transgene and its relevant regulatory regions, (genomic context vectors [GCVs], reaching ultimate complexity in the form of an artificial chromosome [AC]) as vector for the transgene. Appropriate regulation in space and time would be achieved by the presence of the endogenous promoter and other control elements, while retention in daughter cells could be ensured by the presence of sequences which guarantee episomal replication.

In this review, we describe recent advances in GCVs and ACs and the technology underlying their construction. These vectors have been shown to be suitable for delivery and expression of therapeutically relevant genes, including CFTR. The major issue which now limits their routine use is delivery inefficiency. Once this issue is resolved, we will be closer to achieving the goal of regulated gene therapy for CF.

Keywords: Genomic context vectors, artificial chromosome, pulmonary disease, adeno-associated virus, lentivirus, airway epithelium, host defences.

INTRODUCTION

Cystic Fibrosis (CF, OMIM# 219700) is the most common life-shortening autosomal recessive disorder in Caucasian populations and its clinical symptoms are the consequence of mutations in the CF transmembrane conductance regulator (CFTR) gene on chromosome 7q31.2. The gene, and the corresponding mRNA, are both relatively large, the gene spanning 250 kb and the mRNA 6.5 kb, of which 4440 bases is amino acid coding sequence. The gene consists of a TATA-less promoter and 27 exons.

The CFTR gene encodes a transmembrane glycoprotein of 1480 amino acids and has a calculated molecular mass of 168 kDa [Riordan *et al.* 1989]. CFTR resides on the apical membrane of epithelial cells lining the airways, biliary tree, intestines, vas deferens, sweat ducts and pancreatic ducts [Tsui 1995]. CFTR is a member of the ATP binding cassette family of transporters, although uniquely it is formed of five domains: two membrane spanning domains each composed of six subunits; two nucleotide binding domains; and a cytoplasmic regulatory domain. The membrane spanning domains appear to contribute to the formation of a chloride channel pore, since mutation of specific residues within the first membrane spanning domain alters the anion selectivity of the channel [Rich *et al.* 1990; Welsh and Smith 1993]. The nucleotide binding domain of CFTR is responsible for the binding and hydrolysis of ATP and provides the energy necessary for channel activity [Tsui 1995]. The regulatory domain modulates the channel activity of CFTR and can have both inhibitory and stimulatory effects [Rich and Berger 1993].

Although over 1200 mutations and 200 polymorphisms in the gene have been described so far, one mutation, ΔF508, predominates in Caucasian population (Kerem *et al.*, 1989). This three base pair deletion results in the absence of phenylalanine at residue 508 of the CFTR protein and is present in approximately 70 percent of defective CFTR alleles and in 90 percent of patients.

Different classes of gene mutation leading to CFTR defects have been identified: absence of synthesis (class I); defective protein maturation and premature degradation (class II), to which ΔF508 belongs; disordered regulation, such as diminished ATP binding and hydrolysis (class III); defective chloride conductance or channel gating (class IV); a reduced number of CFTR transcripts due to a promoter or

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splicing abnormality (class V); and accelerated turnover from the cell surface (class VI) [Zeitlin 2000; Rowe *et al.* 2005]. Class 1–3 mutations are most common and are associated with pancreatic insufficiency, whereas patients with rarer class 4–6 mutations typically do not have this defect [The cystic fibrosis genotype-phenotype consortium 1993].

Accumulating evidence has demonstrated that CFTR is a cAMP dependent chloride channel [Abakas 2000]. However, this function alone cannot explain the entire pathology of the disease. Numerous other functions have been described that do not relate directly to a disease mechanism based on a channelopathy [Mehta 2005]. The list of proteins with which CFTR interacts and its role as a conductance regulator continues to grow and includes channels (sodium, potassium and calcium-activated chloride), transporters (ATP and glutathione), and proteins linked to the apical cytoskeleton scaffolding of epithelial cells [Mehta 2005]. Although these regulatory roles are not yet fully explained, these findings suggest that the CFTR regulates multiple pathways.

Importantly for what has emerged in the pathophysiology of lung disease, CFTR co-regulates sodium transport through an epithelial sodium channel (ENaC). Wild type CFTR inhibits ENaC sodium transport, while mutant CFTR allows enhanced sodium transport [Stutts *et al.* 1995]. The exact mechanism of this regulation has not been elucidated. The loss of epithelial sodium channel (ENaC) down-regulation in the apical membrane of airway epithelia by mutant CFTR results in increased absorption of sodium ions and fluid across airway epithelia, which depletes the periciliary liquid layer and ultimately depresses the mechanical process of mucus transport. Failure of the mechanical clearance function of the innate defence system brought about by abnormal ion transport therefore leads directly to the chronic cycles of intralumenal airway infection and inflammation that characterize CF lung disease [Knowles and Boucher 2002]. Recently, a mouse model overexpressing the β subunit of ENaC has been characterised, displaying some of the features of CF lung diasease (i.e. submucosal gland hyperplasia, depletion of airway surface fluid, hypersusceptibility to bacterial infection) [Mall *et al.* 2004]. All these studies highlight possible therapeutic targets for CF disease that could lead to treatments addressing the primary (correcting CFTR mutations) or the secondary defects (restoration of proper channel and transporter activities) responsible for the clinical manifestations of CF.

CFTR exhibits complex pattern of tissue-specific expression being expressed at low levels in specialized epithelial cells of gut, airways, pancreas, sweat gland ducts and the male reproductive tract [Crawford *et al.* 1991; Trezise and Buchwald 1991; Engelhardt *et al.* 1992; Trezise *et al.* 1992]. In the airways, CFTR expression depends on the cell type: high levels have been found in serous cells of submucosal glands [Engelhardt *et al.* 1994]. A more recent study [Kreda *et al.* 2005] showed that significant levels of CFTR are found in the apical plasma membrane of all ciliated epithelial cells in the superficial epithelium, and at the apical surface of ciliated cells in submucosal gland ducts. Although CFTR gene expression was initially thought to be dictated by an 'housekeeping' promoter, it turns out that regulation of expression of the CFTR gene involves the interaction of a number of different regulatory factors and elements. Cell-specific expression is determined by regulatory elements found upstream and downstream of the coding region and in various introns [Smith *et al.* 2000]. A regulatory element was located within a DNase I hypersensitivity site 10 kb into the first intron [Smith *et al.* 1996]. This site was seen only in cell lines that transcribe CFTR mRNA and the relative degree of hypersensitivity correlated with the relative levels of endogenous CFTR expression. Distict, tissue-specific patterns of CFTR start site usage were identified in both mouse and human [White *et al.* 1998]. Single transcription start sites were mapped up to –549 and –132 in mouse and human, respectively. A novel 5'-untranslanted exon of murine *Cftr*, denoted exon –1, was identified and shown to be expressed exclusively in mouse testis [White *et al.* 1998]. In addition, the observed patterns of expression indicate that CFTR expression is regulated by hormones [Rochwerger and Buchwald 1993] and during development and differentiation [Trezise *et al.* 1992; Tizzano *et al.* 1993; Trezise *et al.* 1993].

Lung disease is the chief cause of morbidity and mortality in CF patients and current therapies are aimed at controlling the respiratory symptoms by antibiotic and anti-inflammatory treatments [Gibson *et al.* 2003; Ratjen and Doring 2003]. In patients with end-stage lung disease, lung transplantation is the ultimate therapeutic choice.

GENE THERAPY APPROACHES TO CYSTIC FIBROSIS

Gene therapy might prove to be the definitive treatment for CF lung disease. About 30 clinical trials have been carried out in CF patients by using non-viral (cationic lipids) and viral vectors (adenovirus and adeno-associated virus). While both kinds of gene therapy agents have shown proof-of-principle of gene transfer, neither has yet demonstrated persistent expression in human CF nose and lungs, nor provided unequivocal clinical benefit [Bragonzi and Conese 2002; Griesenbach *et al.* 2002]. Two trials using adeno-assocated virus (AAV) were published in the last three years. Flotte *et al.* [Flotte *et al.* 2003] used the promising AAV2-based vectors on 25 CF patients showing very limited gene transfer into either the nose and bronchi and no correction of nasal potential difference. Moreover, at higher viral doses anti-AAV neutralizing antibodies are induced in abundance, posing a serious concern about re-administration of AAV. Mosse *et al.* [Moss *et al.* 2004] published a phase II trial assessing the safety and efficacy of repeated administration of AAV2. Virus administration was safe and, importantly, a significant reduction in sputum interleukin-8 (a neutrophil chemokine) and an improvement in lung function (evaluated as forced expiratory volume (FEV_1)) was seen after the first administration, but not after the second or third administration. Like the previous study, all subjects receiving AAV exhibited an increase (by at least fourfold) in serum AAV2-neutralizing antibodies and detectable levels in bronchoalveolar lavage (BAL) fluid from five of six treated subjects.

The search for new viral vectors belonging to other families has highlighted the potential of Sendai virus (SeV) and lentivirus (LV). Recombinant SeV is a very efficient gene transfer agent for airway epithelial cells partly because it does not need to enter the nucleus [Yonemitsu *et al.* 2000;

Griesenbach *et al.* 2006b]. However, SeV-mediated gene expression is transient and, therefore, requires repeat administration. This is inefficient due to immune responses, and attempts at tolerization against immuno-dominant SeV peptides were unsuccessful [Griesenbach *et al.* 2006a]. LV vectors are integrating and can transduce non-dividing cells, such as those of the respiratory epithelium [Copreni *et al.* 2004]. Unlike SeV, they are inefficient *in vivo* unless there is prior disruption of the epithelial barrier integrity by Ca^{2+}-chelating agents like EGTA [Wang *et al.* 1999; Borok *et al.* 2001] or thixotropic agents [Anson *et al.* 2006]**,** inhalation exposure to sulfur dioxide [Johnson *et al.* 2000], or modification of the barrier function of the airway epithelium with lysophosphatidylcholine (LPC) [Limberis *et al.* 2002]. Although is not known whether any immune response is generated after transduction of the lung with LV vectors, one study reported that *in vivo* delivery of EboZ pseudotyped HIV vector in tracheas of immunocompetent mice did not induce inflammatory infiltrates at any time points post-infection [Kobinger *et al.* 2001]. However, the feasibility of repeat administration has to be demonstrated. Finally, insertional mutagenesis is still a major concern, as for oncoretroviral vectors [Hacein-Bey-Abina *et al.* 2003a; Hacein-Bey-Abina *et al.* 2003b; Fischer *et al.* 2004; Fischer and Cavazzano-Calvo 2005].

Only one non-viral clinical trial has been published in the last three years. A non-viral 'DNA nanoparticle' has been developed, consisting of polyethyleneglycol (PEG)-substituted 30-mer polylysine peptides. This vector mediates gene transfer into the airway epithelium of mice with unprecedentedly high levels and minimal toxicity [Ziady *et al.* 2003a; Ziady *et al.* 2003b]. The DNA nanoparticle's small size (< 20 nm) is thought to facilitate the transport of complex though the nuclear pore. A Phase I clinical trial completed in 2004 found most patients (8 out of 12) had evidence of partial to complete correction of the electrophysiological defect after nasal dosing [Konstan *et al.* 2004].

Even though this is an encouraging result for the development of non-viral vectors for CF gene therapy, limited gene expression is still problematical, and there remains the need for repeat administration without losing efficiency and with minimal toxicity. This is not attainable with current non-viral vectors: improvements centre on the inclusion of alternative promoters [Gill *et al.* 2001], and the removal of proinflammatory and immunostimulatory bacterial sequences, such as CpG motifs [Yew *et al.* 2000].

DRAWBACKS OF CDNA EXPRESSION CASSETTES

Most gene therapy constructs currently in use consist of cDNA expression cassettes driven by strong heterologous and often virally derived promoters. Unfortunately many such transgenes have been found to cease expression after a short period in vivo [Porteous *et al.* 1997]. What causes this to happen? First, it has been established from animal work that cDNA-based transgenes are often subject to transcriptional silencing [Bestor 2000]. This can be alleviated by the insertion of introns and/or the inclusion of flanking insulator elements [Pikaart *et al.* 1998]. Transgenes of unaltered genomic structure in YACs are less prone to silencing [Asselbergs *et al.* 1998], and expression of a transgene from a large BAC vector after transient transfection persists much longer than the same transgene from a small vector [Baker and Cotten 1997]. Second, viral promoters are known to be switched off through the action of cytokines and other molecules [Paillard 1997]. This effect could be negated by the use of host-endogenous promoters. Evidence is accumulating that a transgene containing sufficient genomic context will be expressed for a sustained period [Wade-Martins *et al.* 2003]. The provision of natural genomic context incorporating transcriptional and other control elements has the further advantage that the specificity of expression will more closely resemble that of the normal gene, attributes that are clearly desirable for gene therapy [Vassaux 1999].

STRATEGIES TO OBTAIN GENOMIC CONTEXT VECTORS

The use of genomic fragments containing the genes of interest instead of minigenes based on heterologous promoter and cDNA to supplement mutant genomes with the missing function is at the present one of the major challenges in gene therapy. More than one issue has to be addressed: i) isolation and identification of the genome fragments containing the required information (gene plus regulatory elements); ii) assembly of shuttle vectors that allows isolation and production of such fragments; iii) introduction of the identified locus into the host cells, without causing adverse events due to integration.

The identification of genomic loci takes advantage of the PAC and BAC libraries that have been mainly produced for genome sequencing and that constitute a source of 50-250 kb fragments ordered in contig maps. It is usually possible to identify PAC/BAC vectors spanning the locus of interest by querying suitable databases (e.g., http://bacpac.chori.org/). But since PAC and BAC libraries have been produced by a shotgun approach, it is worth nothing that the locus of interest often needs to be assembled from two or more different vectors according to the contig maps. This may be a difficult and time consuming step, although methods based on Red gene-mediated homologous recombination supplied by defective λ prophage, have been developed to retrofit and modify PAC/BAC vectors [Lee *et al.* 2001; Kotzamanis *et al.* 2005].

A second source of genomic fragments is represented by YAC libraries (http://www.cephb.fr/ceph_yac.html), YAC vectors may be preferred since they harbour larger fragments (an average of 300 kb in size) but are more difficult to handle and isolation of pure YAC DNA is quite inefficient and requires laborious procedures. Nonetheless it is possible to convert YACs into BAC vectors by retrofitting plasmids based on homologous recombination [Cocchia *et al.* 2000].

The human CFTR locus has been identified in the YAC 37AB12 [Anand *et al.* 1991] which is about 320 kb in size, and contains the entire CFTR gene including 70 kb upstream exon 1. The 3' end of the gene was found to be very close to the YAC arm, but the gene is intact, as demonstrated by restriction analysis. The activity of this gene was demonstrated to be tissue specific and to complement CF null mice in vivo. This is the first and the only demonstration of physiological CFTR gene expression mediated by a GCV vector *in vivo* [Manson *et al.* 1997].

A third approach toward the construction of vectors with intact genes is cloning by TAR (transformation associated recombination) which allows isolation of any DNA fragment from complex genomes in the yeast *Saccharomyces cerevisiae*. The procedure makes use of a vector containing specific targeting sequences (hooks, 60-300 bp in size) flanking the locus of interest that promotes insertion of that fragment within the vector. It allows cloning of fragments up to 250-300 kb in two weeks with a yield of positive clones ranging from 1-5%, when intact genomic DNA is used, to 30% when a DSB (double strand break) is induced near to the locus of interest [Kouprina and Larionov 2006].

GENOMIC CONTEXT VECTORS FOR CF GENE THERAPY

Using a novel two-stage recombinational method, a set of PAC-based plasmid GCVs containing chimeric g/cDNA CFTR genes has been generated and characterized, each with 66 kb of 5' genomic sequence that range in size from 85 to 200 kb and vary in the number of introns (0-16) included [Boyd *et al.* 1999a; Walker *et al.* 2004]. GCVs are named rc1-rc15 according to which exon marks the junction of genomic and cDNA (see Fig. **1** for an example). It is important to determine whether such constructs, through inclusion of 5' and intronic control elements, provide more sustained and appropriate expression *in vivo*. Given that introns and untranslated sequences downstream of exon 15 are absent, it is likely that the current set of GCVs will not contain all the necessary signals for tissue specificity: however, they represent useful prototypes that will enable us to begin to dissect the elements important for restricting expression to the epithelial of the lung.

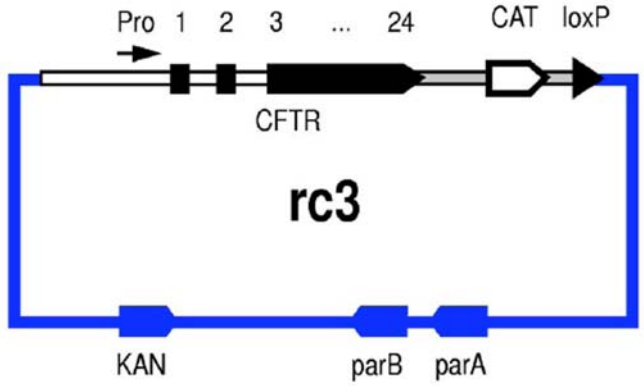

Fig. (1). Schematic drawing of a CFTR-expressing GCV.
CFTR gDNA is shown as an unfilled rectangle with exons represented by solid black boxes numbered 1—3 ... 24 (other exons omitted for clarity). The arrow labelled Pro represents the CFTR promoter. The PAC backbone is shown as a solid line, and PAC markers shown are: parA and parB, partitioning genes; KAN, kanamycin resistance gene. The loxP site is represented by a solid triangle. CAT, chloramphenicol acetyl transferase gene.

Harris *et al.* have intensively studied CFTR gene expression and surveyed DNase hypersensitive sites (DHS) distributed throughout 400 kb of the human CFTR locus [Smith *et al.* 2000]. DHS are the best-characterised indicators of potential transcription regulatory sequences: underlining their importance, high-throughput methods have recently been deployed to catalogue DHS on a genome-wide basis [Crawford *et al.* 2004]. All of the rcN series of GCVs contain the -20.9 kb DHS, already known to be expressed in a tissue specific manner. The GCV with most introns, rc15, contains in addition the DHSs in introns 1, 2, 3, and 10 but not those in introns 16, 17a, 18, 20 and 21 or those 3' of CFTR. While the DHS in intron 1 has a strong association with tissue specificity [Smith *et al.* 1996], it is unknown to what extent other intron DHS are required for *in vivo* tissue specificity. Adding an extra level of complexity to the question of tissue-specificity has been the work of Thomas *et al.*, who by aligning CFTR sequences from many different species identified hundreds of relatively short, highly conserved sequences (multiply conserved sequences) [Thomas *et al.* 2003]. While many of these coincide with coding sequence and known DHS, many do not: at present the function of these other sequences is unknown. However, it is likely that some MCS are involved in the control of tissue specificity.

Qualitative RT-PCR analysis of GCV-transfected epithelial and other cell lines known either to express or not to express endogenous CFTR (including COS-7 (simian kidney, $CFTR^-$), 14HBEo- (human bronchial epithelial, $CFTR^+$), CFBE41o- (human bronchial epithelial, $CFTR^-$), MDCK (canine kidney epithelial, $CFTR^+$), Caco2 (colon carcinoma epithelial, $CFTR^+$), A549 (human alveolar epithelial carcinoma, $CFTR^+$) and primary sheep tracheal epithelial cells) have demonstrated GCV-specific CFTR mRNA expression [Boyd *et al.* 1999a; Boyd *et al.* 1999b]. Furthermore, the DNA sequence of coding regions and associated splicing control elements has been verified for rc7. However, so far we have been unable to assess the contribution of individual introns to cell or tissue specificity because of the difficulty of achieving consistent sufficiently high levels of transfection in established or (especially) primary airway epithelial cells with such large plasmids, despite adopting published methods [Baker and Cotten 1997; Tucker *et al.* 2003; Magin-Lachmann *et al.* 2004]. Only sporadically are transfection efficiencies greater than about 1% achieved in challenging systems such as 14HBEo- and primary cells.

The aim, therefore, is to improve GCV transfer efficiency to levels at which meaningful comparisons can be made between the contributions of each intron to cell- and tissue-specificity, and also to permit functional studies. To facilitate this, three GCVs have been retrofitted with CMV-lacZ reporter gene expression cassettes to produce rc1b (90 kb), rc7b (150 kb) and rc13b (200 kb). One, rc2, has also been retrofitted with Pcmv-EGFP [Walker *et al.* 2004]. The latter is especially useful as it allows FACS-based detection of transfection, which gives a precise figure for percentage of cells transfected with high sensitivity. One approach being actively explored is to assess the effect of albumin on PEI-mediated transfection of GCVs. Albumin is already known to enhance small plasmid transfection in human airway-derived cell lines [Carrabino *et al.* 2005]. A range of non-viral transfection agents are also being tested.

ARTIFICIAL CHROMOSOMES FOR CF GENE THERAPY

The use of genomic loci containing therapeutic genes is in principle preferable to cDNA constructs since they ensure the presence of the control elements, such as locus control regions, alternative splicing signals, intronic control ele-

ments, normally associated with chromosomal genes which dictate the proper spaciotemporal expression of the genes. But using such DNA fragments in gene therapy approaches is hampered by their size, which exceeds the capacity of conventional virus-based vectors. The GCVs described here have no known mammalian origins of replication, and their fate on entry into the nucleus is unknown. It is possible that (like smaller plasmid-based systems) they may persist for many months as episomes *in vivo* in cells that are terminally differentiated and non-dividing, such as those of the airway epithelium. However, artificial chromosomes (AC), molecules mimicking the actions of natural chromosomes, are undoubtedly the ideal platform for large DNA fragments as they would be capable of being maintained at low copy number and segregate faithfully into daughter cells. Their use as vectors for therapeutic genes has been proposed since 1997, when the first *de novo* chromosome was published [Harrington *et al.* 1997; Ikeno *et al.* 1998]. Artificial chromosome assembly and engineering still requires complex and time consuming techniques that limit the exploitation of ACs as carriers of exogenous genes both *in vitro* and *in vivo*. Two approaches can be used to load *yfl* (*your favourite locus*) into an AC: site specific recombination into a preformed AC, and assembly of a *de novo* chromosomes with *yfl*. We will review the more recent advance in both approaches discussing the major limitations and the problems that need to be further addressed.

Insertion of *yfl* by Cre/loxP site specific recombination was successfully used by a number of authors to insert minigene and/or genomic constructs into preformed AC; more recently Oshimura and coworkers reported insertion of heat-regulated human insulin minigene [Suda *et al.* 2006] and the human HPRT YAC/BAC vector [Ayabe *et al.* 2005] into the 21 pqHAC artificial chromosome, obtained by chromosome fragmentation (Fig. **2**, bottom). In both cases the authors obtained a very high efficiency of appropriate integration which produced 100% insulin-21 pqHAC and 56% HPRT-21 pqHAC; interestingly the two experiments were performed in different cell lines suggesting that the Cre/loxP system is independent from the cellular background and is feasible in many cell type. It is important to point out that the selection system used by Oshimura's group relies on the reconstitution of a functional resistance gene (the Neo gene) obtained by Cre-mediated integration of the pCMV-5'neo-loxP containing construct into the loxP-3'-neo region of the HAC.

ACs containing actively expressing genes can be also obtained by a *de novo* approach which is based on HT1080 (an immortalized human fibroblast cell line) transfection of either natural or synthetic alpha satellite DNA fragments consisting of tandem arrays of the alpha subunit (Fig. **2**, top). The efficiency of *de novo* chromosome formation is variable and depends on the alpha DNA higher order repeat. Although the sequence requirement for *de novo* chromosome assembly is not fully understood, there is good experimental evidence demonstrating a critical role for the Cenp-B box, a 17-bp binding site for the centromeric protein Cenp-B, in the assembly of active centromeric chromatin [Ohzeki *et al.* 2002]. Telomeres seem not to be necessary for *de novo* chromosome formation although telomeric fragments added to the alphoid-constructs have been shown to increase the efficiency of chromosome formation [Ebersole *et al.* 2000]. In any case, irrespective of the presence of proper telomeric DNA, most of the *de novo* chromosomes produced have been shown to be circular. As concern origin of replication (ori), no information is available about the activity of this element in ACs. Although ACs must contain origin/s of replication they have not been mapped yet. Researchers do not pose particular attention to ori since it is generally stated that mammalian origins fire at 50-300 kb intervals (reviewed in [Gilbert 2004]) thus any piece of DNA in this range should contain an ori. It would be of particular interest to determine whether addition of *bona fide* origin of replication (i.e. DHFR and β-globin replicator-initiator fragments) [Aladjem *et al.* 1998; Kalejta *et al.* 1998] could improve AC functions.

When alphoid constructs are co-transfected with gene-containing vectors (Fig. **2** top, right panel) or the alpha DNA arrays and the gene of interest (Fig. **2** top, left panel) are assembled in a single vector (PAC, BAC or YAC backbone),

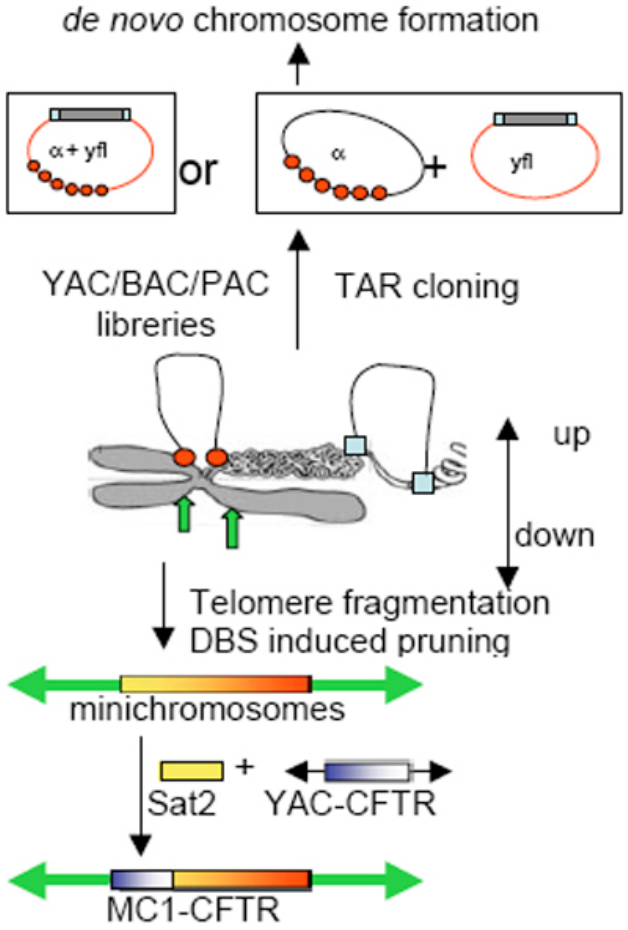

Fig. (2). Schematic representation of the two approaches used to get AC.

Up: *de novo* chromosome formation is obtained by trasfection of vectors containing alphoid DNA (α) and *yfl* (*your favourite locus*) or by co-trasfection of different vectors containing the alphoid and the *yfl* respectively. α- and *yfl*– vectors can be obtained from YAC/BAC/PAC libraries or by TAR cloning. Down: AC obtained from natural chromosomes by telomere fragmentation or by DSB induced breakage. MC1-CFTR was obtained from Sat2 and CFTR-YAC co-transfection.

de novo chromosomes containing the gene of interest are formed. When investigated, it was shown that the resulting AC contains multiple copies of the transgene [Mejia *et al.* 2001] and that it is subjected to physiological regulation [Ikeno *et al.* 2002]. Very recently this approach has been further improved by the assembly of herpes simplex type 1 vectors containing the 17α centromeric DNA, selectable and marker genes (HSV-HAC amplicon) [Moralli *et al.* 2006] which gave rise to AC formation in a panel of different cell types (fibrosarcoma, lung and kidney fibroblast, glioma, primary keratinocyte and primary fetal lung) with an efficiency ranging from 10% (glioma cells) to 90% (primary fetal lung).

A similar HSV-based strategy was pursued to express full-size human genes from BACs. A technology for the rapid conversion of BAC clones into high-capacity HSV-based amplicon vectors has been developed [Wade-Martins *et al.* 2001]. This approach has been used to express the human low-density lipoprotein receptor (LDLR) [Wade-Martins *et al.* 2003] and three cell-cycle regulatory proteins (p16INK4a, p14ARF, p15INK4b) [Inoue *et al.* 2004]. In both cases, the delivery of such genes proved to determine regulated gene expression at physiological levels.

Functional activity of the transgenes cloned into ACs does not seem to be compromised, despite the fact that ACs are small molecules mainly constituted by centromeric chromatin. Many examples of active transgenes cloned in the proximity of centromere and even within centromeric DNA have been reported. HPRT (human hypoxanthine guanine phosphoribosyltransferase) and GCH1 (guanosine triphosphate cyclohydrolase) genes, which have been used in the *de novo* approach, have been demonstrated to be actively transcribed although embedded into centromeric chromatin [Grimes *et al.* 2001; Mejia *et al.* 2001; Ikeno *et al.* 2002]. Of particular interest is the insertion of 300 kb fragment spanning the entire human CFTR gene and including 70 kb 5' upstream, into the pericetromeric region of MC1 [Auriche *et al.* 2002], a human minichromosome of 5.5 Mb obtained from chromosome 1 by a pruning procedure (Fig. **2**, bottom). The insertion was obtained by introduction of the CFTR-YAC (37AB12, [Anand *et al.* 1991]) and pericentromeric Sat2 DNA into MC1 containing cells; 10% of the selected clones were shown to contain the expected integration into the pericentromeric satellite 2 DNA. Analysis of the CFTR transcript, CFTR protein and functional assay by means of eletrophysiological measurements showed the activity of the minichromosome encoded CFTR gene [Auriche *et al.* 2002] demonstrating that gene expression is not affected by the presence of a nearby active centromere. The fact that active genes can be cloned very close or within centromeric DNA is supported by the finding that centromeres are not exclusively composed of closed constitutive heterochromatin but contain distinct chromatin domains consisting of alternating CenpA (the centromeric histone H3 variant) and H3 nucleosomes, the latter having a distinct H3 methylation pattern that distinguishes the centromeric chromatin, characterized by H3 dimethyl Lys 4, from flanking heterochromatin containing H3 methyl Lys 9 [Sullivan and Karpen 2004]. These domains are dynamic structures that do not preclude gene expression and centromeres of artificial chromosomes are organized into dynamic domains as well [Lam *et al.* 2006].

We can definitively state that artificial chromosomes with a defined structure and containing the gene of interest properly equipped with regulatory sequences can be obtained. What is still missing is the development of efficient transfer technique to introduce these huge molecules (1-10 Mb in size) into human cells and model animals. This is a significant limitation since it preclude important functional and preclinical studies in models of human diseases both *in vitro* and *in vivo*. In fact, to date only a few cases of AC transfer to model systems have been reported, the most recent ones being 21DpqHAC-epo, which contains the human *epo* minigene under the control of the CMV promoter. After it was transferred into human primary fibroblasts (hPFs) by microcell-mediated chromosome transfer (MMCT, schematically depicted in Fig. **3**), a number of clones harbouring the minichromosome as a supernumerary element and without detectable chromosome rearragements were isolated and

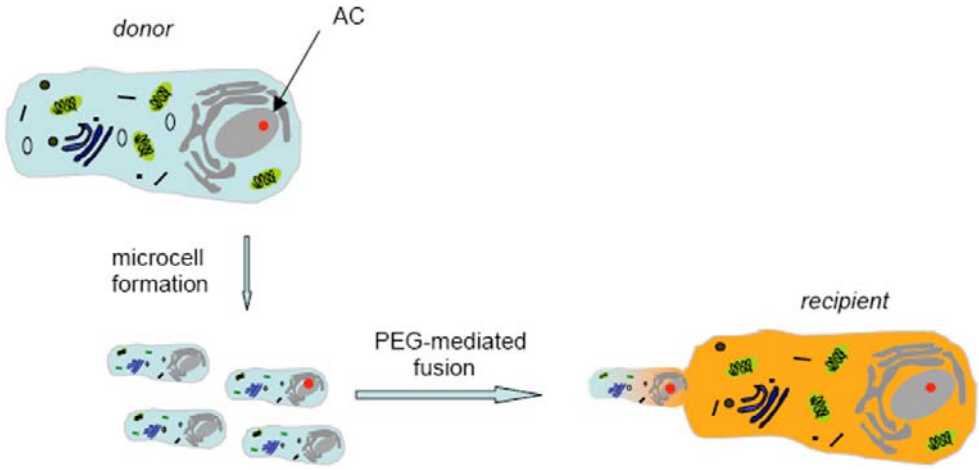

Fig. (3). Schematic representation of microcells mediated chromosome transfer (MMCT) into mammalian cells.
Microcells are produced from donors (e.g. minichromosomes-containing cells) and subsequently fused to new recipients in the presence of polyethyleneglycol (PEG). Selection is subsequently applied to isolate AC-containing cells.

demonstrated to produce human erythropoietin for at least 12 weeks [Kakeda *et al.* 2005]. The same chromosome transfer procedure was used to transfer AC into human hematopoietic and mesenchymal stem cells (MSC) [Ren *et al.* 2005; Yamada *et al.* 2006]. In the latter case the ability of MSC-minichromosome cells to differentiate into osteocytes, adipocytes and condrocytes has been evaluated; moreover cell-lineage specific expression of the reporter gene (EGFP) cloned under the promoter of the osteogenic specific *osteopontin* (OPN) gene was demonstrated.

Only a special class of artificial chromosomes, artificial chromosome expression system (ACEs), that due their large size and characteristic DNA composition can be purified from host chromosomes by dual laser flow sorting, has been transferred to new host cells by commercial cationic agents such as Superfect, TransIT LT and Lipofectamine plus [Vanderbyl *et al.* 2005]. This work demonstrates that, when pure and intact artificial chromosome has been isolated, it may be transfected into new host cells by means of commercial transfection reagents.

Application of AC-based gene therapy requires validation in animal models: two organisms have been used to this purpose: mice, which have been produced by injection of AC-containing ESCs into mice blastocysts [Shen *et al.* 2000; Tomizuka *et al.* 2000; Voet *et al.* 2001] and cows [Kuroiwa *et al.* 2002] which have been produced by using primary fetal fibroblasts in which the AC were transferred by MMCT; cloned calves were produced from Tc (transchromosomic) fibroblast. In both animals, presence of the autonomous chromosomes was demonstrated and the expression of the transgene was properly detected. Moreover, when investigated, it was demonstrated germ line transmission of the AC [Voet *et al.* 2001].

DELIVERY OF GCVS AND ACS

As discussed, the main obstacle for the use of GCVs both *in vitro* and eventually in CF gene therapy is the lack of efficient means of delivery. The CF lung epithelium presents a formidable potential barrier to gene entry that affects all non-viral gene transfer systems. Even once the plasma membrane has been traversed, it has been determined that passive diffusion of DNA >1 kb through cytoplasm is extremely inefficient [Lukacs *et al.* 2000]: it is therefore especially important that reagents for the delivery of GCVs are able to exploit existing cellular trafficking machinery. Indeed, this is a universal problem for non-viral gene therapy and advances in the field generally should be exploitable to facilitate GCV delivery.

Genetically modified and attenuated strains of bacteria have been shown to transfer plasmid DNA to mammalian cells (bactofection) [Palffy *et al.* 2006]. The bacteria used for transfer to phagocytic and nonphagocytic cells are facultative intracellular pathogens that have been engineered to lyse after cell invasion [Grillot-Courvalin *et al.* 1998; Fajac *et al.* 2004]. Proof-of-principle that CFTR cDNA can be transferred and expressed by means of an attenuated strain of *Listeria monocytogenes* has been given [Krusch *et al.* 2002]. Recently, it has been demonstrated that BAC/PAC-based constructs (up to 220 Kb) can be transferred into mammalian cells by an invasive *E. coli* strain in which they can be introduced and propagated resulting in *de novo* formation of ACs providing stable gene expression [Laner *et al.* 2005]. Similarly, a PAC construct of 160 kb containing a large genomic CFTR cassette was stably propagated in a bacterial vector and transferred into HT1080 cells where it was transcribed and correctly spliced, indicating transfer of an intact functional region of at least 80 kb [Laner *et al.* 2005]. The basic mechanism of bactofection and its application to BAC/PAC transfer are shown in Fig. **4**.

AC delivery is even more complicated by the larger size of these molecules although we should distinguish between delivery of chromosomal components (see chapter above) and delivery of functional minichromosomes. In the former case, the same methods used to deliver GCVs can be used, in the latter case the method of choice is undoubtedly MMCT (microcells mediated chromosome transfer) [Killary and Fournier 1995] (Fig. **3**) although lipid-mediated flow sorted minichromosome transfer has been described [Vanderbyl *et al.* 2005].

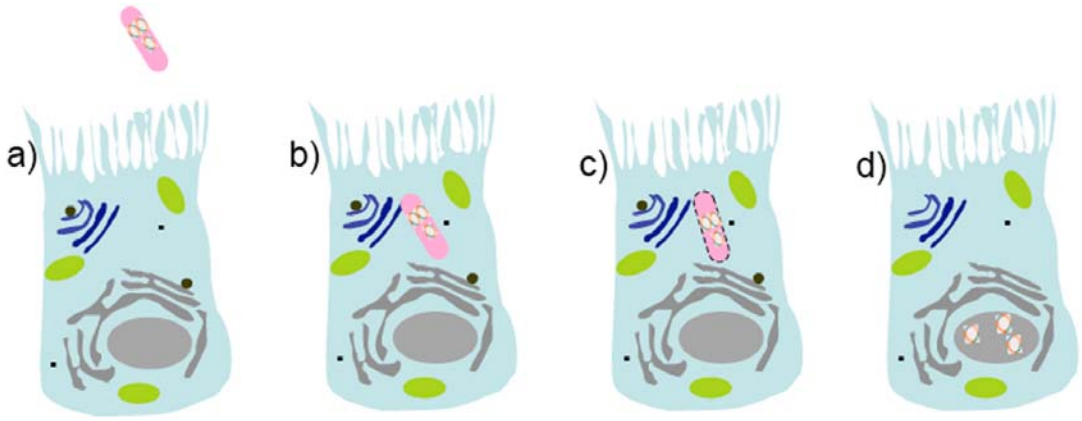

Fig. (4). Bactofection.
E. coli is used as vehicle to transport BAC/PAC into the respiratory epithelial cell (shown as polarized and ciliated). **a**) Transformed bacteria that contain BAC/PAC carrying the CFTR gene are applicated into the airways. **b**) Genetically engineered bacteria penetrate into the cells. **c**) Bacteria undergo lysis induced by the breakdown of lysosomal membranes. **d**) The released BAC/PAC gets into the nucleus and the CFTR gene is expressed by the eukaryotic transcription and translation machinery.

The ideal setting for the treatment of the CF lung disease would be transplantation with bone marrow (BM)-derived stem cells or local stem/progenitor cells *ex-vivo* transfected/transduced with regulated and persistently expressed wild-type CFTR. Although plasticity of this kind of stem cell is still the topic of much debate [Wagers and Weissman 2004], homing to the airway epithelium and transdifferentiation of hematopoietic stem cells transduced with a recombinant retrovirus has been demonstrated [Grove *et al.* 2002]. Both ESCs [Coraux *et al.* 2005] and mesenchymal stem cells (MSCs) from bone marrow [Wang *et al.* 2005] have been shown to become a fully differentiated airway epithelium. Importantly, upon transplantion of CFTR+/+ GFP^+ bone marrow cells into CFTR-/- mice, Bruscia *et al*. demonstrated very low levels of engraftment (0.01-0.1%) in the gut, correlating with very low CFTR mRNA expression [Bruscia *et al.* 2006]. Surprisingly the bioelectric profile of CF mice transplanted with wild type bone marrow was significantly improved in both gut and nose compared to those transplanted with bone marrow from CF mice. This implies that a very low level of cell therapy produced an amplified electrophysiological effect.

Very recently it was demonstrated that an S/MAR-based vector is maintained as an independent molecule in human hematopoietic progenitor cells, although in a small fraction of the population [Papapetrou *et al.* 2006], suggesting that stem cell engineering with non viral vector is feasible. The recent accomplishment of AC transfer into ESCs and MSCs with evidence of correct and regulated gene expression [Shen *et al.* 2000; Tomizuka *et al.* 2000; Voet *et al.* 2001; Ren *et al.* 2005] is a further step on the way to stem cell-mediated gene therapy, though there remain profound conceptual and practical difficulties to achieving this goal. In principle, bone marrow-derived stem cells can be transferred with GCVs or ACs by means of bactofection or MMCT and these cells can be reintroduced into the host (Fig. **5**).

PROSPECTS FOR GCV- AND AC-BASED GENE THERAPY

In our previous review [Conese *et al.* 2004], we predicted some advances in optimization of AC vectors, with particular attention to the definition of the exact molecular composition of the DNA mixtures used to generate *de novo* chromosomes. Indeed, a bimolecular BAC vector system, which is comprised of BAC-GEN and a BAC-CEN, was used to rapidly design, construct and validate multiple *de novo* HACs containing large (100-200 kb) genomic loci including therapeutically significant genes for human growth factor (HGH), polyctsic kidney disease (PDK1) and β-globin [Basu *et al.* 2005].

An efficient viral delivery system would in principle solve the delivery and CpG-related problems. However, while viruses of the correct packaging capacity for GCV-sized transgenes exist (e.g. HSV; [Wade-Martins *et al.* 2003]), their use for CF gene therapy requires considerable development. Firstly, the tropism of such vectors would have to be changed to improve epithelial cell uptake: this could in principle be achieved by pseudotyping [Wilson 2004]. Secondly, and more fundamentally, the immune response to viral envelope proteins that so far thwarts efforts to readminister viruses to lung [Harvey *et al.* 1999] would need to be countered. Whether this major challenge for viral gene therapy could be overcome by chemical immune suppression, or by harnessing viral genes that interfere with the immune response, remains to be seen.

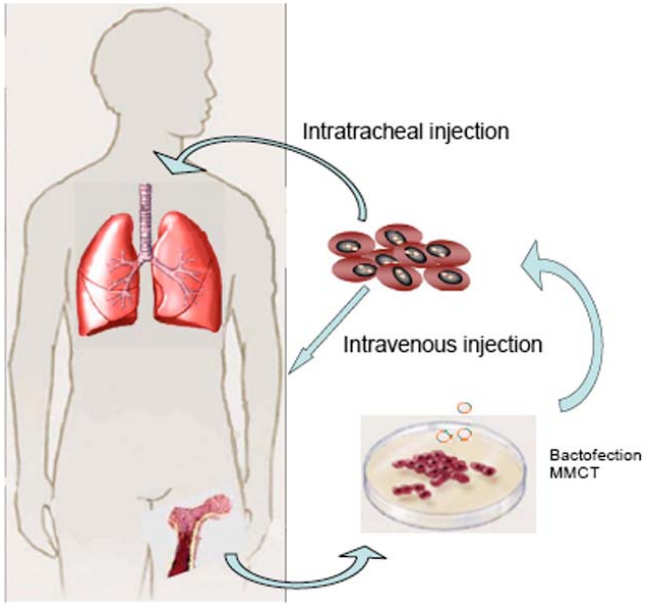

Fig. (5). Schematic drawing of the use of bone marrow-derived stem cells as vehicles of GCVs or ACs to the lungs, target of CF gene therapy.
MSCs can be isolated from bone marrow and transferred *ex-vivo* with GCVs (BAC/PAC constructs) or ACs by means of bactofection or MMCT respectively (see text). The engineered cells are then reinfused into the host by intravenous injection. Intratracheal injection would be preferred to overcome tissue barriers and to target the desidered cellular compartments in the conductiong airways, i.e. the surface epithelium and the submucosal glands.

Non-viral approaches would avoid the readministration problems of viruses. However, GCV use *in vivo* requires the development of synthetic vectors which are efficient in large plasmid delivery. We are confident that such vectors will be developed, and may well exploit receptor-mediated uptake and utilise helper molecules (such as albumin) to optimise delivery.

A novel approach to deliver BACs and PACs to mammalian cells makes use of bacteria as shuttle vectors (bactofection). This approach could be controversial in the context of CF lung disease, which is characterized by bacterial colonization and subsequent infection with opportunistic pathogens [Gibson *et al.* 2003]. However, attenuated bacterial strains, although invasive, might be engineered to be non-pathogenic to CF lung epithelia and, once they have entered into airway cells, programmed to die. Finally, recombinant donor bacteria could be also considered as a way of addressing innate immune responses against CpG motifs.

A CpG-TRIGGERED DEFENCE SYSTEM

A major factor affecting the efficacy of plasmid-based gene transfer to the airways is the presence of a toll-like re-

ceptor 9-mediated cellular defence system triggered by unmethylated CpG motifs [Schwartz *et al.* 1997]. One proven way of circumventing this is to design and synthesize plasmids with highly reduced numbers of CpG motifs [Yew *et al.* 2002]. Clearly, such an approach is much less feasible with plasmids as large as GCVs, but the CpG problem may prove to be intrinsically less significant in GCVs.

The CpG defence system nevertheless poses a major obstacle to non-viral gene therapy. Simply methylating all CpG sequences prior to transfection is not a generic solution, as such treatment abolishes expression from most promoters (e.g. the CMV promoter/enhancer; [McLachlan *et al.* 2000]). The CFTR promoter is rich in CpG sequences and is likely to be switched off by wholesale methylation. However, the system is complex and subtle, with the sequence context of each CpG playing a significant role -some contexts are immunostimulatory and some immunoinhibitory regardless of their methylation status; furthermore, it appears that immunostimulatory motifs can be overcome to some extent by the co-presence of immunoinhibitory sequences [Stacey *et al.* 2003]. Immunoinhibitory sequence contexts appear more frequently in mammalian DNA: it may be, therefore, that GCVs are intrinsically less immunostimulatory because they mainly contain mammalian DNA sequences with only a minor fraction of bacterial DNA. In cases where mammalian context alone is not sufficient for protection, alternative approaches, such as suppressing the CpG defence system, are also worthy of consideration. There has been promising progress in this direction using inhibitory oligonucleotides to suppress TLR signalling for the treatment of lupus [Lenert 2006]: it is reasonable to expect that similar means could be adopted for use in the lung environment.

CONCLUSION

In summary, important progress in the assembly of proper vectors (GCVs and ACs) suitable to carry a CFTR gene construct made to contain its entire coding and regulatory sequences was obtained. However, application of GCVs and ACs requires further exploitation of the delivery methods to improve transfer efficiency of these large DNA molecules into the appropriate target cells, the airway epithelium and/or stem cells of different origins. Nonetheless, we believe that a physiologically regulated gene expression, such as those obtained from GCVs or ACs vectors, will play an important role in the development of gene therapy protocols for CF and other diseases in the next future.

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ABBREVIATIONS

AC = Artificial chromosome

ACE = Artificial chromosome expression system

AAV = Adeno-associated virus

BAC = Bacterial artificial chromosome

BAL = Bronchoalveolar lavage

BM = Bone marrow

CEN = Centromeric

CF = Cystic fibrosis

CFTR = Cystic fibrosis transmembrane conductance regulator

CMV = Cytomegalovirus

DHS = DNase hypersensitive sites

DSB = Double strand break

EboZ = Zaire strain of the Ebola virus

EGFP = Enhanced green fluorescent protein

EGTA = Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid

ENaC = Epithelial sodium channel

ESCs = Embryonic stem cells

FACS = Fluorescent activated cell sorter

FEV = Forced expiratory volume

GCH = Guanosine triphosphate cyclohydrolase

GCV = Genomic context vector

HAC = Human artificial chromosome

HGH = Human growth hormone

HIV = Human immunodeficiency virus

HSV = Herpes simplex virus

HPRT = Hypoxanthine guanine phosphorbosyltransferase

LacZ = Bacterial β-galactosidase gene

LPC = α-L-lysophosphatidylcholine

LDLR = Low density lipoprotein receptor

LV = Lentivirus

MCS = Mesenchymal stem cells

MMCT = Microcell-mediated chromosome transfer

Neo = Neomycin

OMIM = Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM)

OPN = Osteopontin

PAC = P1 artificial chromosome

PDK = Polycistic kidney disease

PEG = Polyethyleneglycol

SeV = Sendai Virus

TAR = Transformation associated recombination

Tc = Transchromosomic

YAC = Yeast artificial chromosome

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