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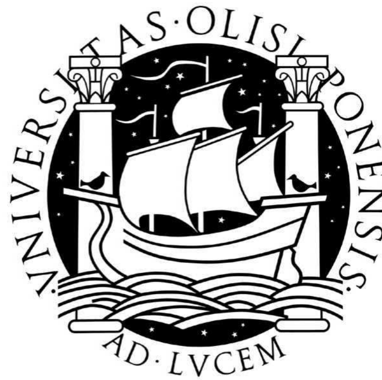

PRODUCTION OF A GENOMIC CFTR CONSTRUCT INSERTED INTO A HUMAN ARTIFICIAL CHROMOSOME (HAC) AND CHARACTERIZATION OF ITS EXPRESSION

Carla Susana Rodrigues Braz

DOUTORAMENTO EM BIOQUÍMICA
(Genética Molecular)

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Tese orientada pela Prof. Doutora Margarida D. Amaral

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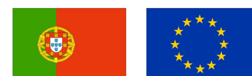

De acordo com o disposto no artigo 41.º do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, Deliberação nº 1506/2006, publicada no Diário da república, 2.ª série — N.º 209 — 30 de Outubro de 2006, foram utilizados nesta dissertação resultados do seguinte artigo:

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No cumprimento do disposto na referida deliberação, esclareço serem da minha responsabilidade a execução das experiências que estiveram na base dos resultados apresentados (excepto quando referido em contrário), bem como a sua interpretação, discussão e redacção.

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PREFACE

Cystic fibrosis (CF), the most lethal genetic disease among caucasians is caused by mutations in the CFTR gene, which encodes the cystic fibrosis transmembrane conductance regulator (CFTR) protein. This makes CF a monogenetic disease and simultaneously a good candidate for gene therapy.

The focus of my research was not the CFTR protein but the gene that underlies it and the assembly of a human artificial chromosome (HAC) that could be used as a gene therapy tool for CF. Like the protein it encodes, the CFTR gene is not an easy one. The gene promoter has no TATA box and resembles, in its characteristics, a house keeping gene. However, it is regulated both temporally and spatially, an indication of complexity. Definitely, that gene and its regulatory sequences still have many secrets for scientists to uncover.

Let us now speak of gene therapy for CF, the main cause behind this work. Gene therapy is a science field still in its infancy, where scientists are groping their way in search of answers, it is a path full of difficulties. At the same time, anyone who believes seriously in gene therapy knows that quitting is out of the question, as the ones who are working in this field right now are aware that they are laying the foundations for a future cure of CF. As is mentioned in the General Introduction to this thesis, the CF disorder can be caused by a great variability of different mutations (the CF data base records over 1700 mutations and the number keeps rising), so it is going to be difficult to find a drug that is able to provide cure (and not just an alleviation of symptoms) for all cases of disease. Gene therapy, which implies the delivery of a normal copy of the CFTR gene to affected cells, on the other hand, is a treatment independent of any specific mutation, which makes it an all encompassing tool against the multimutation, life-shortening CF disease. However, the target of CF gene therapy, the airway epithelium, is well protected against foreign invaders (which may include vectors carrying therapeutic genes) by the mucociliary clearance system and the glycocalyx. Although these barriers are somehow weakened in CF patients, due to the characteristics of the disease, the delivery of a transgene by a gene therapy vector is further hindered, in the case of the CF lung, by the presence of a thick, dehydrated mucus inherent to the disease.

Yes, there is a huge amount of hard work ahead of gene therapy researchers but as I said above we simply cannot quit because more than just a research field, gene therapy is a question of humanity. This work is a humble step towards the

advancement of gene therapy and human artificial chromosomes and its parts shall be described below.

Chapter I is divided into two main sections. In section I an overview of CF and the CFTR gene and protein is given. Starting with an historical background regarding the identification of the CF disease, we then make a description of the disorder and its underlying gene and protein. We describe briefly CFTR protein function and how it regulates other channels and transporters, like a lord of the channels. Next for this multifunctional protein its proposed role as a putative Pseudomonas aeruginosa receptor is mentioned. A description of CFTR mutations, namely the most prevalent one, F508del, follows. Finally, an account of the pharmacological approaches towards a treatment of CF is given. Section II provides a small description of gene therapy for CF and gene delivery systems, including both viral and non viral vectors. Chapter I finishes by alluding to the centromere as an essential component in any human artificial chromosome.

Chapter II includes the results. A thorough description of the assembly of the fusion PAC CF225, carrying a reconstructed CFTR locus, followed by the analysis of its stability is made. Next comes the precise identification of the genomic insert/vector junctions as well as the two deletions in introns 9 and 10, which led to the subsequent determination of the length of the reconstructed locus. The results of co-transfecting a lung sarcoma cell line with CF225 and the TTE1 construct carrying a selectable marker and centromere to form a de novo human artificial chromosome (HAC) are described at the end of the chapter.

In Chapter III an overall discussion of this work is given. Chapter IV gives a description of the materials and methods used in this study. In Chapter V preliminary assays to construct a pre-fabricated HAC are mentioned and strategies for the continuation of the work are suggested. A pre-fabricated HAC carrying a copy of the CFTR gene and its regulatory sequences is essential to attain a CF cure through gene therapy.

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O caminho continua…

SUMMARY

Cystic Fibrosis is an autosomal recessive disorder, which makes it a good candidate for gene therapy. Thus cystic fibrosis became one of the first targets for gene therapy since apparently it is sufficient to deliver a normal copy of the gene encoding the CFTR protein to the affected cells.

Although promising at first, classical gene therapy to cystic fibrosis has not met expectations due to immune response against viral vectors and synthetic vectors, as well as short-term expression of cDNA based transgenes. These hurdles can be overcome by delivering the complete genomic *CFTR* gene on non-integrating human artificial chromosomes (HACs). Here, we describe the reconstruction of the genomic *CFTR* locus into one P1-based artificial chromosome (PAC), CF225. This is a non-selectable PAC of 225.3 kb (running from -60.7 kb to +9.8 kb) which resulted from the ligation of two PACs, CF1-Met and CF6, with an optimized M470V codon and a silent *Xma*I restriction variant to discriminate transgene from endogenous expression. CF225 was shown to be stably maintained and propagated in the *E. coli* DH10B host. After co-transfection of CF225 with the telomerized, blasticidin-S selectable, centromere-proficient alpha satellite (cen 5) TTE1 construct into HT1080 fibrosarcoma lung cells, CF225 was not incorporated into a *de novo* HAC in 122 lines analyzed, but five integrants formed, four of which expressed the transgene, as detected by RT-PCR and *Xma*I restriction analyses. Stability analyses suggest feasibility to pre-fabricate a large, tagged *CFTR* transgene that stably replicates in the proximity of a functional centromere. Although definite conclusions about HAC proficient construct configurations cannot be drawn at this stage, an important transfer resource was generated and characterized, demonstrating promise of *de novo* HACs as potentially ideal gene-therapy vector systems.

Keywords: Cystic Fibrosis, CFTR, Gene Therapy, Human Artificial Chromosome (HAC), P1 Based-Artificial Chromosome (PAC)

RESUMO

A terapia génica pode ser definida como a introdução de um gene exógeno numa célula receptora para a obtenção de benefícios terapêuticos. Os objectivos a longo prazo dos estudos relacionados com a terapia génica são o desenvolvimento de vectores como ferramentas para o estudo do genoma e da função cromossómica e para transferir genes para as células com fins terapêuticos.

A Fibrose Quística é uma doença autossómica recessiva, o que implica que uma única cópia normal do gene *CFTR*, que codifica a proteína do mesmo nome, é suficiente para restaurar a função CFTR de canal de cloreto ausente em caso de mutação em ambos os alelos *CFTR*. Estas características tornam a Fibrose Quística um candidato atraente para a terapia génica, visto bastar transferir uma cópia do gene normal para as células afectadas para se obter a cura da doença. Assim, pouco tempo após a descoberta do gene *CFTR*, em 1989, tiveram início os primeiros esforços no sentido de tornar a terapia génica da Fibrose Quística uma realidade.

Contudo, a terapia génica da Fibrose Quística tem como requisitos que o gene terapêutico deva ser expresso em todas as células epiteliais onde normalmente ocorre expressão, de preferência a níveis comparáveis aos do gene endógeno. Além disso, a expressão deverá ser estável e persistente, a fim de ser evitada a readministração repetida do gene terapêutico.

A maioria dos vectores utilizados actualmente em terapia génica consiste em cassetes de expressão controlada por promotores heterólogos fortes, frequentemente derivados de vírus. Contudo, descobriu-se que muitos destes transgenes são expressos apenas durante um período de tempo limitado. A capacidade dos vírus para infectar as vias respiratórias fez deles a escolha natural inicial para a terapia génica da Fibrose Quística e muitos dos primeiros estudos foram efectuados com vectores virais derivados de adenovírus.

A expressão do gene *CFTR* é regulada espaciotemporalmente, pelo que a sua estabilidade a longo prazo e expressão regulada de forma tecido-específica requerem não só o promotor e as porções codificantes do gene como também elementos cromossómicos reguladores, tais como os estimuladores e silenciadores, associados aos locais hipersensíveis à DNase I (DHS, na terminologia inglesa). Estes requisitos exigem a utilização de vectores de grande capacidade, como é o caso de vectores cromossómicos, tais como os cromossomas artificiais bacterianos

(BACs), os cromossomas artificiais baseados no fago P1 (PACs) e os cromossomas artificias humanos (HACs).

Há duas vantagens essenciais dos sistemas baseados em vectores cromossómicos em relação à maior parte dos vectores convencionais usados para transferência génica. Em primeiro lugar, o DNA transferido pode ser mantido estavelmente sem os riscos associados à inserção e, em segundo lugar, podem ser introduzidos grandes segmentos de DNA englobando os genes e os seus elementos reguladores, conduzindo à expressão do transgene mais fiável e fisiológica, mais próxima da do gene normal. Além disso, os vectores cromossómicos constituídos apenas por DNA humano não deverão ser imunogénicos.

Tratando-se da Fibrose Quística, o epitélio das vias respiratórias é o alvo mais importante, uma vez que a doença pulmonar é a que contribui principalmente para a morbilidade e a mortalidade. Dado que esta doença, pelo menos nas etapas iniciais, afecta essencialmente as vias respiratórias inferiores, é provável que as células epiteliais destas vias sejam importantes, sendo consideradas por muitos autores como as células alvo apropriadas para a terapia génica da Fibrose Quística.

Após a produção do constructo genómico CGT21 que contém aproximadamente metade do locus do gene *CFTR* e o último exão e da demonstração de que ele era propagado estavelmente em células de sarcoma do pulmão, onde era expresso e sofria *splicing* correcto (Laner et al., 2005), o passo lógico seguinte era gerar um constructo *CFTR* genómico portador dos 27 exões e das sequências reguladoras flanqueantes para incorporação num HAC.

O objectivo consistia na reconstituição do gene *CFTR* completo clonado num PAC [o PAC usado neste trabalho foi o pCYPAC2 (Ioannou et al., 1994)] adequado para a preparação em grande escala de DNA de elevada qualidade. Nós descrevemos aqui a construção de um locus *CFTR* contendo os 27 exões e a maior parte das potenciais regiões reguladoras num só CF PAC. Partindo de preparações de DNA armazenadas em *plugs* de agarose e previamente caracterizadas (Ramalho et al., 2004), contendo parte do gene *CFTR* em PACs, nós construímos o PAC CF225, portador do gene *CFTR* humano com todos os exões e intrões mais sequências reguladoras, ligando dois PACs, CF1-Met e CF6, contendo cada um aproximadamente metade do gene *CFTR* e regiões flanqueantes: o PAC CF1-Met é portador de um inserto que vai de -60,7 kb a montante do início da tradução no exão 1 até ao intrão 10, e o PAC CF6 é portador de um inserto que vai do intrão 10 até +9,8 kb a jusante (relativamente ao fim da tradução). Devido à escolha dos PACs originais, os intrões 9 e 10 estão substancialmente encurtados em 5,1 kb e 27,1 kb, respectivamente. Como consequência, os locais hipersensíveis à DNase I (DHS)

descritos no intrão 10 (McCarthy and Harris, 2005) estão excluídos do locus CF225. Contudo, este clone tem a vantagem de conter o codão da metionina (M) optimizado no locus polimórfico M470V, em comparação com os clones *wild type*, e uma variante de um local de restrição *Xma*I silencioso sintético, o qual é adequado para discriminar entre os produtos de RT-PCR do transgene e os provenientes dos loci *CFTR* endógenos de qualquer célula alvo.

A fim de concretizar um dos objectivos do presente trabalho, partimos de três clones PAC, CF1-Met10-43/-44/-54, cujo exão 10 tinha sido modificado para conter metionina (M) em lugar da valina (V) na posição 470, bem como um local de restrição para a enzima *Xma*I, introduzido por uma mutação silenciosa. O conteúdo destes clones foi sujeito a diferentes análises antes da construção de CF225: 1) amplificações por PCR cujos produtos abarcavam toda a região do exão 10 clonada bem como sequências *wild-type* localizadas a montante e a jusante, as quais mostraram que os tamanhos dos produtos de PCR estavam de acordo com o esperado nos três clones; 2) sequenciação do exão 10 clonado, que confirmou a orientação correcta, a presença do polimorfismo corrigido V470M e que a sequência nos três clones não possuía qualquer mutação derivada de PCR; 3) análise de restrição, tendo revelado que as bandas esperadas correspondiam aos locais de restrição sintéticos *Xma*I e *Not*I; 4) digestão de 1/10 de uma *plug* de agarose dos três clones CF1-Met com as enzimas de restrição *Not*I e/ou *Bss*HII também mostrou os tamanhos esperados para os fragmentos obtidos; 5) os tamanhos esperados para as bandas foram demonstrados para os três clones por reacções de PCR de longo alcance (LR-PCR, segundo a terminologia inglesa) de baixo número de ciclos, cobrindo toda a sequência CFTR clonada nos PACs CF1-Met, incluindo a redução de 5,1 kb no intrão 9. Estes dados indicam que os clones continham o inserto completo sem rearranjos. Como a banda de 31,8 kb do subclone CF1-Met10-43 era muito fraca em comparação com os produtos dos outros subclones bacterianos, indicando possivelmente uma alteração em algumas das bactérias usadas na preparação das *plugs*, o clone correspondente não foi mais usado.

Depois de confirmar que a sequência *CFTR* clonada no PAC CF1-Met tinha a mesma estrutura que a clonada no PAC CF1 original, o passo seguinte era fundir o PAC CF1-Met com o inserto do PAC CF6, o qual é portador do resto da sequência genómica de *CFTR* com as sequências dos exões e das junções exão/intrão correctas. A estratégia de clonagem foi a seguinte: 1) digestão parcial do PAC CF1-Met (clone 44) com *Not*I, digestão total de CF6 com *Not*I seguido por desfosforilação para impedir a recircularização do vector; 2) separação dos produtos de digestão por electroforese em gel de campo pulsado (PFGE, de acordo com a terminologia

inglesa) e excisão das bandas do gel sem exposição a UV; 3) electroeluição dos fragmentos de DNA existentes nos pedaços do gel e sua mistura numa razão de ~1/1 (CF1-Met/CF6); ligação do DNA pela ligase T4; 5) electroporação do produto de ligação em *E. coli* DH10B; 6) selecção, por PCR, das colónias resistentes à kanamicina com os primers CFi10fus/R7, específicos da região de fusão entre os dois PACs. Foi identificado um clone, mais tarde designado por CF225, positivo para a reacção de PCR relativa à região de fusão e para STSs dos exões 4 e 12, presentes nos dois PACs que foram ligados.

Após a identificação do clone positivo, o passo seguinte deveria ser a análise da sua estabilidade enquanto clone. Assim, durante o crescimento inicial da cultura mãe das bactérias portadoras do PAC CF225, foram plaqueados doze subclones individuais e analisados por PCR para avaliação da estabilidade da clonagem. Nove dos doze subclones continham os oito STSs testados por PCR e que cobriam o locus em diferentes posições ao longo do gene *CFTR*, incluindo sequências localizadas a montante do início da transcrição, a região da fusão entre os dois PACs e a região poli (A). Para analisar mais detalhadamente a estabilidade de clonagem, três culturas derivadas de clones individuais do PAC CF225 foram crescidas durante vários períodos de tempo, simulando um rendimento final potencial de 10^{15}, 10^{21} e 10^{27} células de *E. coli*, contendo cada uma até ~10 cópias totalmente replicadas do constructo. A digestão do material contido em *plugs* de agarose, proveniente das colónias derivadas dos três subclones individuais, mostrou a presença, em todos os casos, de um inserto com o tamanho de 225 kb (*Sal*I) e fragmentos idênticos e com os tamanhos previstos (*Bss*HII), indicando uma estabilidade de clonagem global elevada para o locus. Os dados indicavam também a propagação fiel e estável do locus *CFTR* no PAC CF225 (inserto de 225,3 kb), demonstrando que o gene *CFTR* pode ser clonado de forma estável em *E. coli*.

Ainda continuando a análise estrutural do constructo, seguiu-se a sequenciação das suas extremidades, em que o inserto *CFTR* se liga ao vector. Assim, para determinar os extremos de CF225, foram efectuadas reacções de LR-PCR com primers que se ligam nas extremidades 5’ e 3’ da junção inserto/vector. Os fragmentos amplificados foram sequenciados e comparados por meio do programa BLAST com o hg built 37.1 no site NCBI. O locus *CFTR* reconstruído vai do nucleótido -60651 relativamente ao início da tradução ao nucleótido +9767 relativamente ao final da tradução. Ambas as extremidades coincidem com um local de restrição para *Sau*3AI, o que está de acordo com o facto de, para a preparação da biblioteca RPCIP704, para a qual foi utilizado o PAC pCYPAC2, que serviu de

fonte para a construção de CF225, o DNA genómico ter sido parcialmente digerido e clonado no local *Bam*HI do vector.

Como resultado do procedimento da clonagem, CF225 tem duas deleções nos intrões 9 e 10 as quais representam regiões que não estavam incluídas nos PACs originais CF1 (intrão 9) e CF6 (intrão 10) e que foram omitidas pela reconstrução do exão 10 e das suas sequências intrónicas flanqueantes. Para localizar com precisão e determinar a extensão daquelas deleções, foram realizadas reacções de PCR com primers que hibridam em regiões que flanqueiam as duas deleções. Os produtos de PCR foram sequenciados e comparados por meio do programa BLAST com BACs portadores de sequências genómicas humanas publicadas. Ambas as deleções foram localizadas com precisão, tendo a deleção no intrão 9 5058 pb, ao passo que a deleção no intrão 10 tem 27128 pb.

Para conseguir a incorporação do locus *CFTR* num cromossoma humano artificial (HAC, na terminologia inglesa) formado *de novo*, foram efectuadas experiências de co-transfecção do locus *CFTR* clonado com um constructo linearizado portador de sequência de DNA alfa-satélite do centrómero e a expressão dos clones celulares obtidos analisada. Quatro ensaios independentes de co-lipofecção do inserto de 225 kb do PAC CF225 com o constructo TTE1 (fragmento de 133 kb) contendo um gene marcador de resistência à blasticidina S (BS) duplicado e o marcador EGFP, bem como sequências centroméricas, resultaram em 185 clones celulares, 122 dos quais foram expandidos e analisados por PCR com primers específicos para CF225. Cinco clones celulares individuais, BW24, BG32, CG13, DG27 e DG5 eram positivos para a reacção de PCR específica, indicando que apenas 1 em ~25 clones foram co-transfectados em simultâneo com CF225 e TTE1.

Foram realizadas reacções de RT-PCR com primers que geram um produto entre os exões 8 e 10 que, após *splicing*, tem 391 pb e representa uma mistura de produtos dos genes *CFTR* endógenos da linha celular HT1080 e do locus transgénico. Como resultado destas experiências, verificou-se que quatro (BG32, CG13, DG27 e DG5) das cinco linhas co-transfectadas expressavam o locus transgénico. Todas as linhas que expressavam evidenciavam níveis variáveis de expressão CFTR após 30 dias de crescimento sem selecção. Para distinguir entre a expressão endógena e a do transgene, os produtos de RT-PCR foram clivados com *Xma*I que digere o exão 10 modificado de CF225 em dois fragmentos de 310 pb e 81 pb. Nas quatro linhas celulares, proporções variáveis do transcripto CFTR resultavam do transgene, o que demonstrava, em muitos casos, níveis de expressão do transgene acima dos dos genes endógenos (cujos produtos de RT-PCR não

eram clivados por *Xma*I) e mostravam a ocorrência de *splicing* correcto. As linhas celulares que expressavam e as células HT1080 parentais foram analisadas por sequenciação dos produtos de RT-PCR obtidos com os mesmos primers e também com o primer CFc3F (exão 3), demonstrando que todas as linhas continham tanto o polimorfismo 470M como a variante sintética *Xma*I no exão 10, confirmando que tinham origem no transgene.

Para verificar a integridade do constructo CF225 nas linhas celulares clonais, foram realizadas reacções de PCR com primers para a junção vector/CFTR a 5' e para a junção vector/CFTR a 3'. Das cinco linhas celulares derivadas de HT1080 apenas DG27 manteve a extremidade 5' de CF225, confirmada pela sequenciação do produto de PCR. Todas as 5 linhas foram negativas para a reacção de PCR relativa à extremidade 3', bem como para duas outras reacções de LR-PCR que abrangiam aproximadamente 2 kb e 3 kb desde a extremidade 3' do vector até ao locus *CFTR*, sugerindo que o DNA de CF225 a 3' foi perdido em todas as quatro linhas que expressavam.

A fim de averiguar se se tinha formado um cromossoma *de novo* ou se havia ocorrido integração no genoma da célula, foram efectuados ensaios de FISH de tripla cor nestas linhas celulares após 30 dias de crescimento com e sem selecção por BS. Estas análises revelaram ou integração do locus CF225 num cromossoma do hospedeiro ou integração e truncação em todas as cinco linhas celulares clonais. Não se observaram HACs portadores do locus *CFTR*. A linha clonal DG27 mostrou co-integração estável próximo do gene *CFTR* endógeno no cromossoma 7. A linha BG32 revelou uma integração distal/telomérica num cromossoma que não o 7. A linha celular DG5 mostrou a integração de sinais de CF1, CF6 e E1 (centrómero) numa posição distal do cr19q e a linha CG13 mostrou integração de porções de CF1 e CF6 no braço p de um cromossoma metacêntrico que não o 7, acompanhada por truncação. Na linha celular BW24 apenas foram detectadas sequências de CF6 num pequeno cromossoma truncado. No geral, podemos concluir que CF225 e o centrómero E1 não formaram eficientemente em conjunto uma estrutura de replicação estável. Em vez disso, foram seleccionados raros clones estáveis que continham pelo menos o marcador BS e várias porções do locus CF225 sem a extremidade 3', que todavia mostraram expressão e *splicing* correcto da sequência do exão 10 em 4 das 5 linhas obtidas.

No presente trabalho foram também feitas tentativas de construção de um HAC *de novo* (pré-fabricado) por uma abordagem de recombinação *in vitro* por meio de digestão enzimática e ligação, seguida de electroporação em *E. coli*, de um constructo portador do locus CF225 e de TTE1, contendo sequências de DNA

centromérico. Escolheu-se uma abordagem de recombinação *in vitro* para a construção porque esta técnica é mais reprodutível e menos propensa a rearranjos do que as abordagens de recombinação *in vivo*. Não foi possível obter o HAC pré-fabricado e é sugerida uma estratégia para uma futura obtenção do mesmo. A produção de um HAC *de novo*, com todas as vantagens que este tem sobre os vectores virais, é muito importante para o desenvolvimento de uma terapia génica de sucesso para o tratamento da Fibrose Quística. Este trabalho representa mais um passo em frente no sentido de tornar a terapia génica para a Fibrose Quística uma realidade e os resultados obtidos aqui deixam antever a possibilidade de criação de um cromossoma artificial humano portador do gene *CFTR* e respectivas sequências reguladoras o qual representaria um vector ideal e uma promessa de cura para a Fibrose Quística, independentemente da mutação causadora de doença.

Palavras-chave: Fibrose Quística, CFTR, Terapia Génica, Cromossoma Artificial Humano (HAC), Cromossoma Artificial Baseado no Fago P1 (PAC)

ABBREVIATIONS

A	Adenine (base) residue; alanine (amino acid) residue
aa	Amino acid
AAV	Adeno-associated virus
ABC	ATP-binding cassette
ACH	Active chromatin hub
Ad	Adenovirus
ASL	Airway surface liquid
ATP	Adenosine-5'- triphosphate
BAC	Bacterial artificial chromosome
bp	Base pairs
BS	Blasticidin S
C	Cytosine residue
C-terminus	Carboxyl terminus
cAMP	Cyclic adenosine 3',5'-monophosphate
cDNA	mRNA-complementary DNA
cen	Centromere
CENP-A	Centromere protein A
CENP-B	Centromere protein B
CF	Cystic fibrosis
CF HAE	Human CF ciliated surface airway epithelium
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CIP	Calf intestinal phosphatase
CTCF	CCCTC-binding factor
D	Aspartic acid residue
DEAC	Diethylaminocoumarine
del	Deletion
DHS	DNase I-hypersensitive site
DMEM	Dulbecco's modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
dUTP	Deoxyuridine triphosphate
EBNA1	Epstein-Barr virus nuclear antigen 1
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetraacetic acid
EGFP	Enhanced green fluorescence protein

ENaC	Epithelial sodium channel
ER	Endoplasmic reticulum
F	Phenylalanine residue
FCS	Fetal calf serum
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
G	Guanine (base) residue; Glycine (amino acid) residue
GCH1	Guanosine triphosphate cyclohydrolase 1
GDS	Gene delivery systems
HAC	Human artificial chromosome
HAE	Human airway epithelium
HPRT	Hypoxanthine guanine phosphoribosyltransferase
HSP	Heparan sulfate proteoglycan
HSV	Herpes simplex virus
I	Isoleucine residue
IBMX	3-isobutyl-1-methylxanthine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K	Lysine residue
kb	Kilobase (1000 base pairs)
LB	Luria Bertani medium
LINE	Long interspersed element
LMP	Low melting point
LPS	Lipopolysaccharide
LR-PCR	Long range-PCR
M	Methionine residue
MC	Minichromosome
MMCT	Microcell-mediated chromosome transfer
MSD1 and MSD2	Membrane spanning domain 1 and 2
N	Amino terminus; asparagine residue
NBD1 and NBD2	Nucleotide binding domain 1 and 2
NHERF-1/-2	Na^+/H^+ exchanger regulatory factor isoform-1/-2
NPD	Nasal potential difference
ORCC	Outwardly rectifying Cl^- channel
oriP	Epstein-Barr virus origin of replication
P	Proline residue
PAC	P1 phage-based artificial chromosome

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEI	Polyethylenimine
PFGE	Pulsed field gel electrophoresis
PGK	Phosphoglycerine kinase
PIV	Human parainfluenza virus
PKA	Protein kinase A
PKC	Protein kinase C
PLGA	Poly lactic-*co*-glycolic acid
RD	Regulatory domain
RNA	Ribonucleic acid
ROMK	Renal outer medullary K^+ channel
RT-PCT	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SeV	Sendai virus
SINE	Short interspersed element
Sp1	Specificity protein 1
STS	Sequence-Tagged Sites
T	Thymidine (base) residue; threonine residue (amino acid)
TAE	Tris/acetate/EDTA buffer
Taq	*Thermus aquaticus*
Tris	Tris(hydroxymethyl)aminomethane
TM	Transmembrane segments
UTR	Untranslated region
UV	Ultraviolet
V	Valine residue
W	Tryptophan residue
wt	Wild-type
X	Any amino acid residue
YAC	Yeast artificial chromosome

CHAPTER I

GENERAL INTRODUCTION

CHAPTER I. GENERAL INTRODUCTION

I. OVERVIEW OF CYSTIC FIBROSIS (CF) AND THE CFTR GENE AND PROTEIN

I.1. CYSTIC FIBROSIS

I.1.1 HISTORICAL BACKGROUND

Cystic fibrosis (CF) was first recognized as a separate disease from celiac syndrome in 1938 by Dr. Dorothy Hansine Andersen (1901-1963) based on autopsy studies of malnourished infants, which allowed to distinguish a disease of mucus plugging of the glandular ducts, termed “cystic fibrosis of the pancreas” (Andersen, 1938). This thick, sticky mucus clogging the ducts of mucus glands throughout the body gave rise to the alternative designation “mucoviscidosis” (state of thick mucus) (Farber, 1944).

An important discovery was made during the 1948 heat wave in New York by Dr. Paul di Sant’Agnese (1914-2005) who was the first to recognize that babies with CF were at increased risk for heat prostration. This observation led to his discovery that the sweat is abnormal in patients with CF, presenting a fivefold excess of sodium and chloride, which persisted after the heat wave subsided (di Sant'Agnese et al., 1953). Standardization of the sweat test, which became the primary diagnostic test, in 1959 (and still in use today) allowed identification of milder cases, and CF was found to be not only a disorder of mucus (Davis, 2006).

Investigations proceeded in the CF field and in the 1980’s major breakthroughs were accomplished. Thus, in 1983, Paul Quinton, studying the ducts of sweat glands, identified chloride (Cl^-) transport as the basic defect in CF (Quinton, 1983). In another line of investigation, Michael Knowles and Richard Boucher found diminished chloride movement from epithelia into the airway lumen, accompanied by increased sodium reabsorption in the epithelium (Knowles et al., 1983; Boucher et al., 1986).

The discovery of the CF gene by positional cloning, in 1989, resulted from the joint efforts of three research groups, those of Lap-Chee Tsui (Kerem et al., 1989) and Jack Riordan (Riordan et al., 1989) at the Hospital for Sick Children in Toronto, and Francis Collins (Rommens et al., 1989) at the University of Michigan. The discovery of the CF gene led to the demonstration that the impaired chloride transport is due to the failure of a cAMP-regulated Cl^- channel. Since then,

substantial progress in basic and clinical research raised the median survival age of CF patients to ~37 years (Cohn et al., 2005; Wang et al., 2005; Davis, 2006). After 1989, CF diagnosis could also be made by direct identification of two mutant alleles, namely for borderline cases detected by the sweat test (Davis, 2006).

I.1.2 DESCRIPTION OF THE DISEASE

CF (MIM no. 219700), the most common life-threatening genetic disease of Caucasians (Welsh et al., 2001) is inherited in a Mendelian autosomal recessive pattern (Andersen and Hodges, 1946). The disease is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR; ABCC7). This anion selective ion channel is required for the normal function of epithelia lining the airways, intestinal tract as well as ducts in the pancreas, salivary and sweat glands (Ameen et al., 2007) and the absence of its activity results in the failure of ionic and water homeostasis at exocrine epithelial surfaces (Riordan, 2008).

While practically all exocrine glands are affected in CF, the three main organs of greatest clinical importance are the sweat gland (diagnosis), the pancreas (malnutrition), and the lung (morbidity/mortality) (Quinton, 2007).

Clinically, CF disease is characterized by exocrine pancreatic insufficiency, due to failure of bicarbonate-rich fluid and enzyme secretion which impair intestinal digestion and absorption. CF also causes an increase in sweat NaCl concentration [3-5 times higher than in unaffected subjects (Shwachman and Antonowicz, 1962; di Sant'Agnese and Powell, 1962)] and male infertility. However, the major cause of morbidity and mortality is pulmonary disease due to recurrent bacterial infections. In fact, the pulmonary manifestations of CF are responsible for more than 90% of CF-related mortality (Pilewski and Frizzell, 1999).

Significant reduction (>90%) of functional CFTR in the plasma membrane of airway epithelial cells results in a defect in Cl^- secretion, hyperabsorption of sodium and other changes that reduce the capacity of cilia to clear bacteria from the airways (Gibson et al., 2003; Boucher, 2004). In the lung, there is generation of thick and dehydrated mucus and subsequent chronic bacterial infections (mainly by *Pseudomonas aeruginosa*) which lead to bronchiectasis.

The loss of CFTR from airway epithelial cells also leads to altered regulation of other ion channels (e.g., ENaC), significant changes in the composition of airway surface liquid (ASL) (Boucher, 2003) and production of pro-inflammatory cytokines

(Terheggen-Lagro et al., 2005; Machen, 2006). The ultimate destruction of the CF lung is mainly due to inflammation (Chmiel and Davis, 2003).

The frequency of the disease varies among ethnic groups and is highest in individuals of Northern European origin, of which about 1 in 2500 newborns is affected. Similarly, in this population the heterozygote frequency reaches the rather remarkable value of about 1 in 25 individuals (Collins, 1992).

In Portugal, the estimated CF incidence is 1 in 6000 newborns (Farrell, 2008). Recently, in one study by Lemos and colleagues (Lemos et al., 2010), the CF incidence in the central region of the country was calculated as 1 in 14000 newborns, i.e., lower than in the country as a whole.

I.2 THE *CFTR* GENE

The CF transmembrane conductance regulator (*CFTR*) gene is located on the long arm of chromosome 7, at the region 7q31.2 (Kerem et al., 1989;Riordan et al., 1989;Rommens et al., 1989). The gene, and the corresponding mRNA, are both relatively large, spanning ~190 kb and 6129 bp, respectively (Collins, 1992), including 5’ and 3’ untranslated regions (UTR), of which 4443 bases are amino acid coding sequence. The gene consists of a TATA-less promoter and 27 exons (Figure I.1), whose sizes vary greatly from 38 bp (exon 14b) to 724 bp (exon 13) (Zielenski et al., 1991). It comprises 26 introns ranging in size from 600 bp (intron 22) to 28085 bp (intron 10).

I.2.1 THE *CFTR* GENE PROMOTER AND TISSUE-SPECIFIC *CFTR* EXPRESSION

As the characteristics of the *CFTR* gene, namely the elements that drive its expression, are important for gene therapy, a detailed description of the locus will be given here.

Promoter deletion experiments have defined the minimal promoter as -226 to +98 with respect to the transcription start site (+1) at -132 bp 5’ to the first methionine codon (Chou et al., 1991). Position +1, defined by Riordan and co-workers (1989) in their original description of the *CFTR* cDNA, is located 121 bp upstream of the ATG translation initiation codon and corresponds to position +11 in the numbering system used by Chou and co-workers (1991).

The *CFTR* gene has different transcription start sites which vary among cell lines expressing it. In one study (Koh et al., 1993) using the numbering system of Riordan and co-workers (1989), it was found that low abundance transcripts initiate at position -32, while the start site + 50 appears to be the major initiation point for *CFTR* transcription in highly expressing cell lines.

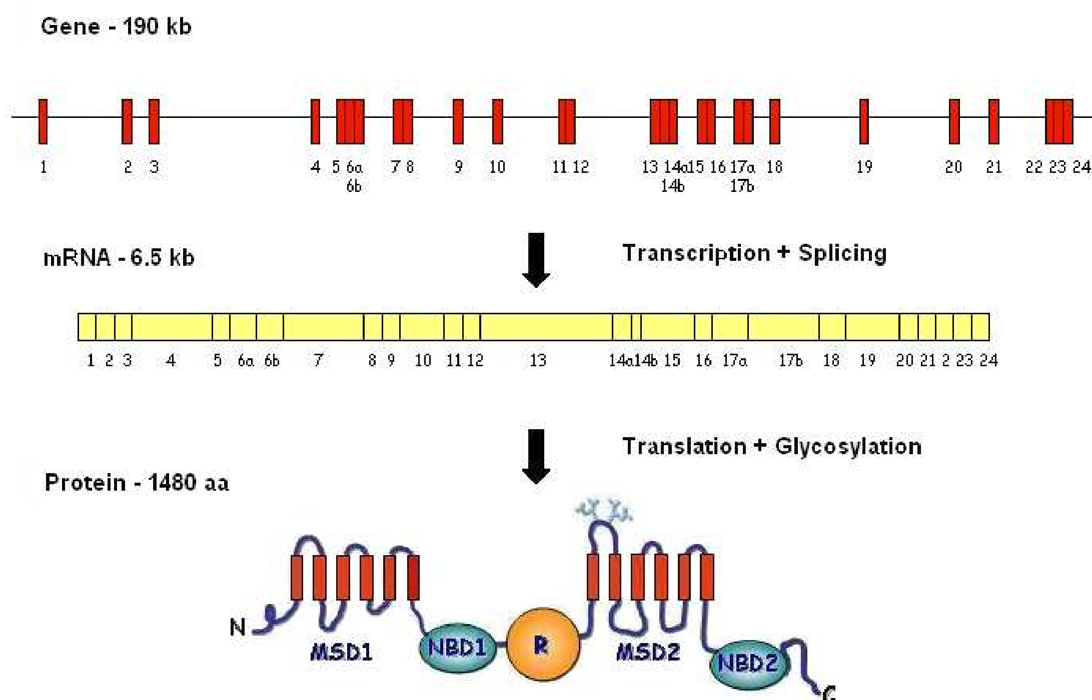

Figure I.1 Scheme illustrating the *CFTR* gene, mRNA, and protein. MSD – membrane-spanning domain (MSDs 1 and 2); NBD – nucleotide-binding domain (NBDs 1 and 2); R – regulatory domain; N – amino terminal; C – carboxyl terminal; aa – amino acid residue. [Adapted from (Zielenski and Tsui, 1995) by MD Amaral].

CFTR exhibits tightly regulated expression, both temporally during development and spatially in different tissue types (Crawford et al., 1991; Trezise et al., 1993; Broackes-Carter et al., 2002). However, the *CFTR* promoter resembles that of a housekeeping gene: it is CpG-rich, contains no TATA box, has multiple transcription start sites and has several putative binding sites for the transcription factor Sp1 [Specificity protein 1; (Yoshimura et al., 1991)]. Consistent with promoters of this type, the *CFTR* promoter is weak and demonstrates no apparent tissue-specificity, suggesting the involvement of distal regulatory elements in control of *CFTR* expression. These elements are associated with DHS (DNase I hypersensitive sites) within the genomic region encompassing the *CFTR* locus, both upstream (at -20.9 kb) and downstream of the coding region (at +5.4, +6.8, +7.0, +7.4 and +15.6 kb) and in various introns (Smith et al., 2000; Phylactides et al., 2002).

A cell-type-specific DHS was identified within the first intron of *CFTR*, at 185 + 10 kb (where 185 is the last base in *CFTR* exon 1) (Smith et al., 1996), which corresponds to a regulatory element that functions as a classical, tissue-specific enhancer and can also independently recruit general factors necessary for transcription initiation (Smith et al., 1996; Mogayzel, Jr. and Ashlock, 2000; Rowntree et al., 2001; Ott et al., 2009b; Ott et al., 2009c). This element was shown to positively regulate CFTR promoter activity specifically in intestinal cells both *in vitro* and *in vivo* (Rowntree et al., 2001).

Blackledge and colleagues (Blackledge et al., 2007) identified two enhancer-blocking insulators, also DHS, located upstream and downstream to the *CFTR* gene that have distinct properties. The insulator located at -20.9 kb from the *CFTR* translation start site was associated with a classical CTCF (CCCTC-binding factor)-dependent insulator element. A second element, located 3' to *CFTR*, within a DHS at +15.6 kb (with respect to the translational end point) also demonstrated enhancer-blocking activity but this was independent of CTCF binding.

Recently, two works (Blackledge et al., 2009; Ott et al., 2009a) reported structural and functional evidence for a *CFTR* transcriptional hub in which intronic enhancer elements are brought into close proximity to the *CFTR* promoter to activate cell-type-specific transcription (Figure I.2). This complex looped structure of the *CFTR* locus occurs in cells that express the gene and is absent from cells in which the gene is inactive. Close interaction of the *CFTR* promoter with sequences in the middle of the gene about 100 kb from the promoter and with regions 3' to the locus that are about 200 kb downstream was demonstrated. These interacting regions correspond to prominent DHS within the locus, which recruit proteins that modify chromatin structure (Ott et al., 2009a).

Other features of the *CFTR* promoter, which may contribute to both the temporal and spatial regulation of gene expression, are the use of multiple transcription start sites for the gene (Yoshimura et al., 1991; Koh et al., 1993) and the recruitment of alternative upstream exons (Broackes-Carter et al., 2002; Mouchel et al., 2003; Lewandowska et al., 2009). Lewandowska and colleagues (Lewandowska et al., 2009) identified a novel *cis*-acting element that contributes to the activity of the basal *CFTR* promoter in airway epithelial cells and showed that a combination of epigenetic modifications contribute to the multiple mechanisms regulating the promoter of the *CFTR* gene.

CFTR exhibits a complex pattern of tissue-specific expression being expressed at low levels [in normal individuals, *CFTR* mRNA transcripts are expressed at 1-2 copies per cell (Trapnell et al., 1991)] 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).

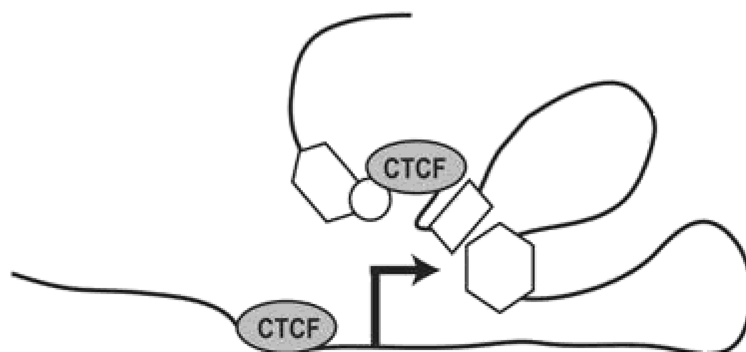

Figure I.2 A looping model for the active *CFTR* gene. In *CFTR*-expressing cell types, elements in the *CFTR* 3' flanking region are in close proximity to the CFTR promoter. This 3' flanking region includes the tissue-specific +6.8 kb DHS, shown here to bind CTCF, as well as other previously described DHS (Nuthall et al., 1999; Blackledge et al., 2007). Protein factors bound at each of these sites interact with the promoter-bound transcription machinery, thus forming an active chromatin hub (ACH) and helping regulate expression of the *CFTR* gene. Besides the DHS from the 3' flanking region, intronic DHS such as the intestine-specific intron 1 element and others may also contribute to the *CFTR* ACH in a tissue-specific manner (Ott et al., 2009b).

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.

I.3 THE CFTR PROTEIN

The product of the *CFTR* gene is a transmembrane glycoprotein of 1480 amino acids (Riordan et al., 1989) that functions as a plasma membrane chloride (Cl^-) channel activated by cyclic AMP (cAMP).

CFTR plays an important role in both secretion and reabsorption of ions and fluid at epithelial surfaces, depending on the electrochemical gradient present. To perform this task, i.e., to respond to cAMP-stimulation following phosphorylation by protein kinase A (PKA) and protein kinase C (PKC), it should be correctly localized at the lumen-facing or apical membrane of epithelial cells (Riordan, 1993).

I.3.1 CFTR: A MULTIDOMAIN MEMBRANE PROTEIN

CFTR consists of 2 repeated motifs, each composed of a hydrophobic membrane spanning domain (MSD1 and MSD2) containing six helices [transmembrane segments (TM)] each which compose the core structure of the pore (Tabcharani et al., 1991), and a cytosolic hydrophilic region for binding with ATP, that is, nucleotide binding domain [NBD1 and NBD2 (Riordan, 1993)]. These 2 motifs are linked by a cytoplasmic regulatory domain (RD), which contains a number of charged residues and multiple consensus phosphorylation sites (substrates for PKA and PKC) (Figure I.3).

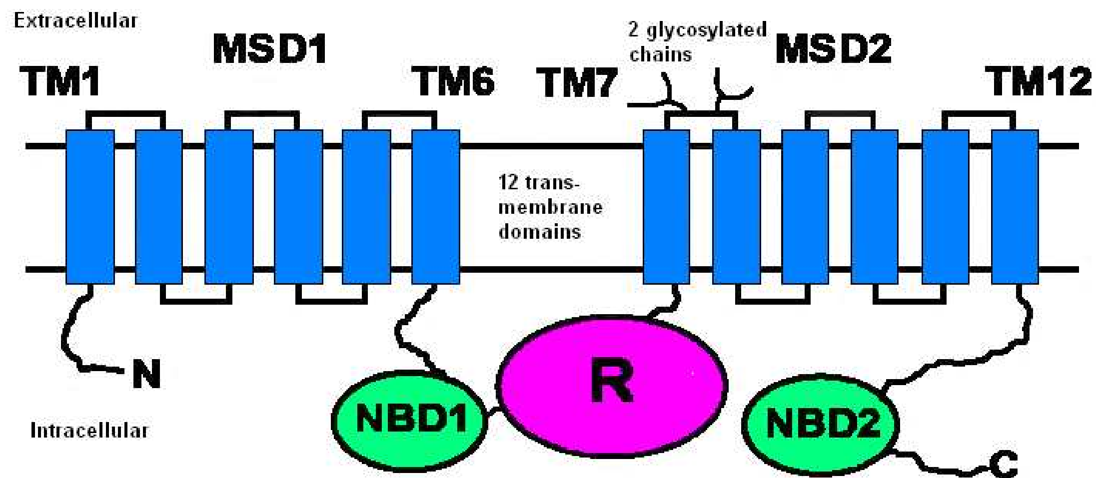

Figure I.3 Simplified topological model of the CFTR chloride channel. The channel is anchored through the membrane with 12 linked membrane spanning domains (MSD) interrupted between the sixth and seventh domains by an intracellular nucleotide binding domain (NBD1) and a putative "regulatory" domain (R). A second intracellular nucleotide binding domain (NBD2) occurs near the C terminus. During processing of the protein, two glycosylated side chains are added to the mature protein to the extracellular loop between transmembrane domains (TM) seven and eight. [Adapted from (Chen and Hwang, 2008)].

I.3.1.1 THE ABC SUPERFAMILY

CFTR, or ABCC7, is a member of the superfamily of ATP-binding cassette (ABC) transporters, the largest class of proteins encoded by the human genome (Amaral, 2006). The family name ABC was applied to reflect the presence in all members of two homologous NBDs (Holland et al., 2003). In many ABC transporters, both of the ATP-binding sites are hydrolytic, whereas in others, including the human ABCC subfamily, to which CFTR belongs, hydrolysis occurs at only one of the sites, in the case of CFTR, at NBD2 (Aleksandrov et al., 2002).

In humans, 48 ABC proteins, grouped into 7 different classes, have been identified (Klein et al., 1999; Dean and Annilo, 2005). CFTR is unique among ABC transporters because it has a RD that is phosphorylated by PKA and PKC. CFTR is functionally distinct from the other ABC transporters because it permits bidirectional permeation of anions rather than vectorial transport of solutes. This adaptation of the ABC transporter structure can be rationalized by considering CFTR as a hydrolysable-ligand-gated channel with cytoplasmic ATP as ligand (Riordan, 2005; Riordan, 2008).

I.3.2 MOLECULAR FUNCTION

Based on the high NaCl concentration in the sweat of CF patients, the first cellular defect demonstrated in CF was found in the sweat duct, which proved to be impermeable to Cl^- (Quinton, 2007). Consistently, the most documented function of the normal CFTR protein is that of an anion conducting channel. Patch-clamp studies have established that the single channel has an anion selectivity pattern of $Cl^- > I^- > Br^- > NO_3^- > HCO_3^-$ >gluconate (Gray et al., 1989; Berger et al., 1991; Linsdell et al., 1997).

CFTR is also reported to be involved in the function or regulation of a number of other channels, transporters and mechanisms (see section I.5.1 for more details).

I.4 CFTR FUNCTION AS A CHLORIDE/BICARBONATE CHANNEL

On the basis of current knowledge only the CFTR protein is required to form an ATP- and PKA-dependent low-conductance Cl^- channel of the type present in the apical membrane of many epithelial cells (Bear et al., 1992).

CFTR mediates transepithelial salt and water secretion into the lumen of kidney tubules, pancreatic ducts, and the intestine (Guggino and Stanton, 2006).

In the sweat duct the opposite mechanism occurs. The CFTR anion channel normally is expressed abundantly in the luminal membrane of the absorptive duct, where it absorbs salt (Cohn et al., 1991; Kartner et al., 1992). Thus CFTR provides for passive conductance of Cl^- ions during reabsorption from the lumen back into the extracellular fluid across the cell. During absorption Na^+ sets up an electrical driving force for the movement of Cl^-. That is, Na^+ passively enters the duct cell from the lumen down its electrochemical gradient through the epithelial Na^+ channel (ENaC) in

the apical membrane (Quinton, 1981). Simultaneously, the transport of positive charge through the cells creates sufficient electrochemical gradients for transcellular electroconductive transport of Cl^- from the lumen through CFTR in the apical membrane and then again through CFTR in the basal membrane to the serosa. Since the sweat duct is one of the few epithelia of the body that is relatively water-impermeable, as Na^+ and Cl^- leave the duct, water cannot follow, and a steep osmotic gradient develops across the duct that parallels the absorption of salt (Quinton, 2007). Thus CFTR plays a major role in preventing the body from losing too much salt during perspiration.

In CF, the reabsorptive mechanism fails due to the lack of functioning CFTR (Quinton, 1983; Quinton and Bijman, 1983). When the CFTR anion channel is absent or inactive, Cl^- cannot follow Na^+ out of the lumen, and both Na^+ and Cl^- absorption are impeded, an effect understood in terms of electroneutrality. If Cl^- cannot be removed from the lumen, an equivalent of Na^+ must remain with it. Thus, in CF patients, neither Cl^- nor Na^+ can be effectively reabsorbed from the duct, and salty sweat appears on the skin surface (Quinton, 2007).

In CF, the exocrine pancreas produces too little HCO_3^-, whose transport also fails in this disease. This causes macromolecules and enzymes (which, under normal conditions, should be diluted and kept inactive) to aggregate and block the small ducts so premature proteolysis and inflammation destroys individual units until the exocrine pancreas becomes inadequate for normal digestion (Hadom et al., 1968; Johansen et al., 1968).

I.5 OTHER CFTR FUNCTIONS – ONE CHANNEL TO RULE THEM ALL

I.5.1 CFTR AS A REGULATOR OF OTHER CHANNELS AND TRANSPORTERS

In addition to its role as a secretory Cl^- channel in epithelial cells, CFTR also regulates several transport proteins, including the epithelial sodium channel, ENaC (Stutts et al., 1997; Ji et al., 2000; Jiang et al., 2000), the outwardly rectifying Cl^- channels, ORCCs (Gabriel et al., 1993; Jovov et al., 1995; Schwiebert et al., 1995; Schwiebert et al., 1999), renal outer medullary K^+ channels, such as ROMK1 and ROMK2 (Yoo et al., 2004) or inwardly rectifying K^+ channels (Schwiebert et al., 1999), ATP-release mechanisms (Schwiebert et al., 1995), anion exchangers (Lee et al., 1999; Ko et al., 2004), sodium-bicarbonate transporters (Shumaker et al., 1999),

and aquaporin water channels (Schreiber et al., 1999; Cheung et al., 2003). Figure I.4 illustrates some of CFTR multifunctions.

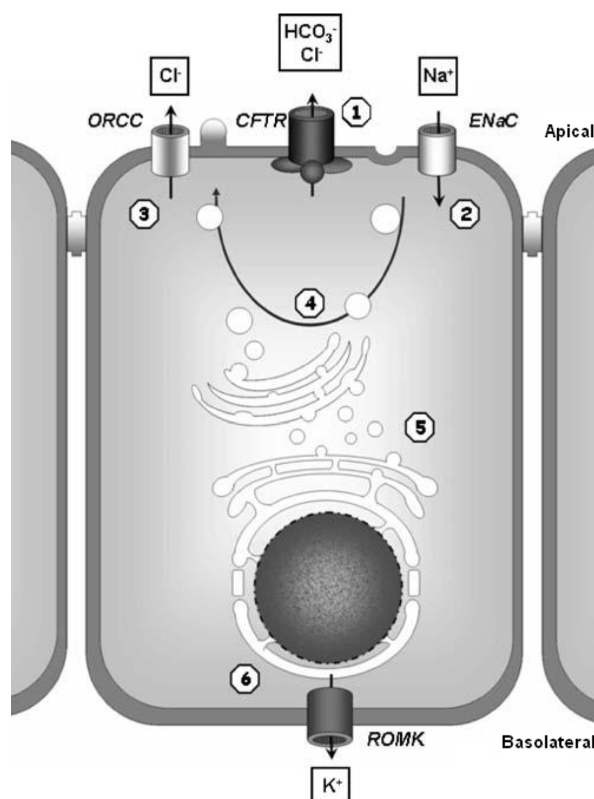

Figure I.4 Overview of CFTR functions and interactions. CFTR functions as a regulator of other ion channels and affects numerous cellular processes: 1) Cl^- channel function, which facilitates the release of Cl^-, HCO_3^-, and ATP, 2) negative regulation of epithelial Na^+ channels (ENaC), 3) positive regulation of outwardly rectifying Cl^- channels (ORCC), 4) regulation of vesicle trafficking, 5) regulation of intracellular compartment acidification and protein processing, 6) modulation of the renal outer medullary potassium channel's (ROMK) sensitivity to sulfonylureas [adapted from (Mueller and Flotte, 2008)].

Importantly for the pathophysiology of CF lung disease, CFTR co-regulates Na^+ transport through an epithelial Na^+ channel, ENaC. Wt-CFTR inhibits ENaC Na^+ transport (except in sweat ducts where CFTR activates ENaC), whereas ENaC activates CFTR, and mutant CFTR allows enhanced Na^+ transport, with a subsequent increase in Na^+ absorption (Stutts et al., 1995; Mall et al., 1996; Reddy et al., 1999; Guggino and Stanton, 2006). This interaction between CFTR and ENaC is biologically relevant because the balance between CFTR-mediated Cl^- secretion and ENaC-mediated Na^+ reabsorption regulates the net amount of salt and water in airway periciliary fluid, and thereby the capacity to clear bacteria and other noxious agents from the lungs (Boucher, 2004).

Wt-CFTR activates the outwardly rectifying Cl^- channels [ORCCs; (Gabriel et al., 1993; Schwiebert et al., 1995; Schwiebert et al., 1999)] through the release of ATP as an agonist into the extracellular milieu. This CFTR-dependent release of ATP out of the cell, allowing interaction with ORCCs, could be conducted by CFTR itself or via a closely associated ATP channel. The incidence of ORCCs has been reported to be enhanced in the presence of a functional CFTR Cl^- channel (Jovov et al., 1995).

Inwardly rectifying K^+ channels (ROMKs) have been identified on the basolateral cell membrane in airway epithelia, where they are believed to play a role in K^+ recycling (Schwiebert et al., 1999). NHERF1 and NHERF2 (Na^+/H^+ exchanger regulatory factor isoform-1/-2) increase the physical interaction between one member of this family, ROMK2 (renal outer medullary K^+ channel) and CFTR (Yoo et al., 2004). The NHERF-facilitated interaction between ROMK2 and CFTR enhances glibenclamide-induced activation of ROMK2.

With CFTR regulating so many channels and processes, the thought "one channel to rule them all" comes to mind.

I.5.2 CFTR AS A PUTATIVE *PSEUDOMONAS AERUGINOSA* RECEPTOR

CF is characterized by the emergence and persistence of (and, ultimately, the inability to clear) chronic infection with a variant of *Pseudomonas aeruginosa* (mucoid *P. aeruginosa)* that over-produces a surface polysaccharide known as alginate, which protects the bacteria from antibiotics and other antimicrobial agents, making the infection very difficult if not even impossible to eradicate (Emerson et al., 2002; Li et al., 2005).

Attachment of *P. aeruginosa* in CF-airways was explained by a mechanism that proposes CFTR as a receptor for *P. aeruginosa* in the airways (Pier et al., 1997), indicating an additional function for the CFTR protein. According to some authors, CFTR is a cellular receptor for binding, endocytosing, and clearing *P. aeruginosa* from the normal lung. Once *P. aeruginosa* is bound to epithelial cells, CFTR accumulates in the cell membrane at a specific point of contact with the bacterial surface. Overall, according to a proposed model (Pier et al., 1997), a specific interaction between *P. aeruginosa* and the first extracellular domain of CFTR triggers CFTR-mediated resistance to infection in individuals who have wt-CFTR. Lack of this interaction and lack of a functional CFTR protein in most CF patients could contribute significantly to the respiratory manifestations of CF.

In CF, the diminished or non-existent binding of *P. aeruginosa* to the CF epithelium leads to a reduced initial clearance, allowing the organisms sufficient time to take advantage of the dehydrated ASL and remain within the airway lumen by binding to mucins via the bacterial FliD protein (Arora et al., 1998). Subsequently increased production of alginate occurs (Worlitzsch et al., 2002; Bragonzi et al., 2005), further serving to protect the microbe from host defences.

Recently, it was shown that *P. aeruginosa* chemically modifies lipid A (contained in bacterial LPS) and muropeptides (contained in peptidoglycan) as a strategy to evade immune system and detection, favouring survival in patients with CF (Cigana et al., 2009).

I.6 CFTR MUTATIONS

To date, more than 1700 variants have been identified in the *CFTR* gene (http://www.genet.sickkids.on.ca/cftr/StatisticsPage.html), most of them causing CF disease.

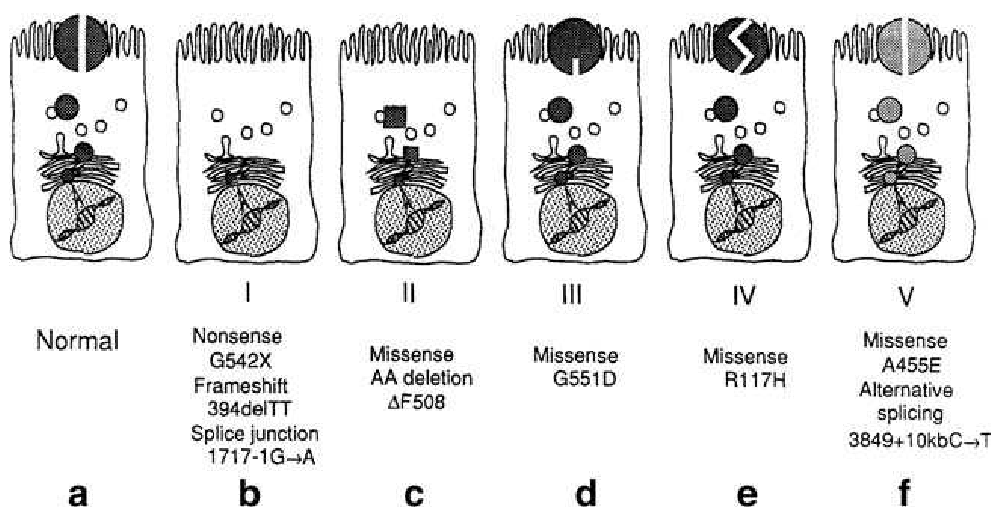

Figure I.5 Molecular consequences of mutations in the *CFTR* gene. a) CFTR protein correctly positioned at the apical membrane of an epithelial cell, functioning as a chloride channel. b) Class I. No CFTR mRNA or no CFTR protein formed (e.g., nonsense, frame shift or splice site mutation). c) Class II. Trafficking defect. CFTR mRNA formed, but protein fails to traffic to cell membrane. d) Class III. Regulation defect. CFTR reaches the plasma membrane but fails to respond to cAMP stimulation. e) Class IV. Channel defect. CFTR functions as an altered chloride channel. f) Class V. Synthesis defect. Reduced synthesis of defective processing of normal CFTR. Chloride channel properties are normal (Proesmans et al., 2008).

Mutations in the *CFTR* gene can be grouped into five different classes according to their effect on CFTR function (Gibson et al., 2003) (Figure I.5). Class I mutations lead to a premature termination codon (PTC) that results in an unstable truncated CFTR transcript and/or no CFTR expression. Missense mutations (Class II), including F508del, cause protein misfolding that leads to the retention of the misfolded protein in the endoplasmic reticulum and premature degradation. Class III mutations result in the reduced capacity of CFTR to secrete Cl^- due to abnormal channel activation by ATP. Class IV mutations cause a reduced capacity to conduct Cl^- across membranes. Class V mutations cause abnormal or alternative splicing, which reduces the amount of functional protein.

I.6.1 F508DEL-CFTR

The most common CF mutation is loss of a phenylalanine (F) residue at position 508 (F508del). Up to 70% of individuals with CF are homozygous for the F508del mutation, and almost 90% of patients may have at least one F508del allele. The finding that F508del is responsible for such a high percentage of all CF mutations suggests that there may have been some heterozygote selection or a very strong founder effect for this particular mutation in the Northern European population (Tsui and Buchwald, 1991; Morral et al., 1994; Alfonso-Sanchez et al., 2010).

The F508del mutation: (a) retains CFTR in the endoplasmic reticulum (ER) where it is subsequently degraded by the proteasome; (b) reduces the capacity of CFTR to transport Cl^- ions (Gibson et al., 2003; Boucher, 2004; Davis, 2006), (c) decreases the plasma membrane half-life of CFTR in polarized human airway epithelial cells (Swiatecka-Urban et al., 2005), and (d) reduces the levels of transcripts (Ramalho et al., 2002).

Most F508del-CFTR protein is rapidly removed from the cell through the cellular disposal machinery (Amaral, 2005). This mechanism substantially prevents F508del from reaching its correct cellular location, the apical membrane of epithelial cells, and explains why this mutation is included in class II (Cheng et al., 1990). Additionally, F508del causes major defects in channel regulation that interfere with channel opening (Wang et al., 2000), and therefore it can also be considered a class III mutation.

In native tissues from F508del-homozygous patients, however, CFTR has been described as having an apical localization (Kalin et al., 1999; Penque et al., 2000),

although the proportion of cells in which this occurs is significantly lower than in wild-type controls (Penque et al., 2000).

Preventing the intracellular retention and degradation of F508del-CFTR, however, would be of great therapeutic importance for the treatment of the CF disorder, as it is believed that once correctly folded, the mutant will traffic to the apical membrane, where it has been reported to retain some function (Denning et al., 1992; Pasyk and Foskett, 1995; Amaral, 2004).

I.6.2 OTHER CFTR MUTATIONS

F508del is the most common CF mutation, but over other 1700 *CFTR* mutations can be found, with frequencies ranging from the relatively high to the rare, sometimes occurring only in one or two families. According to one study (Zielenski and Tsui, 1995), besides F508del, the most common *CFTR* mutations in the world, with relative frequencies above 1%, are 1898+5G->T (30%), 3120+1G->A (11%), I148T (9.1%), G542X (2.4%), G551D (1.6%), N1303K (1.3%), and W1282X (1.2%).

Most of the molecular alterations in the *CFTR* gene are point mutations and, according to the *CFTR* mutation database (www.genet.sickkids.on.ca/), 40.24% are missense mutations, 16.55% are frameshift mutations, 12.49% splicing mutations, and 9.12% nonsense mutations. Both large and small insertions/deletions have also been reported, as well as mutations affecting the promoter of the gene and unknown mutations.

With all this mutational variability affecting the *CFTR* gene, it will be probably hard to find a single cure for CF using drugs. In this respect, gene therapy seems the perfect answer for the treatment of this disease since, by restoring CFTR function through the delivery of a healthy copy of the *CFTR* gene, it would provide a complete cure regardless of the mutation involved, and not just the alleviation of symptoms.

I.7 PHARMACOLOGICAL APPROACHES

CF is still a life threatening disease, the current mean life expectancy of CF patients being ~37 years. Lung disease is the main 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 (Conese et al., 2007;Proesmans et al., 2008). Survival is about 80% at the 1-year mark, and by 4 years is less than 50%, so this is not yet a perfect therapy (Davis, 2006).

While our understanding of CF has increased steadily, the promise of gene therapy is a work in progress, as the current work will show, but not yet a reality. In the meantime, other approaches are being developed to treat CF patients, namely new drugs.

Drug discovery for CF is focused on identifying drugs that allow F508del-CFTR to escape the ER (correctors) and to activate F508del-CFTR channels that reach the plasma membrane (potentiators) (Roomans, 2003; Proesmans et al., 2008; Kreindler, 2010), since it is the most frequent mutant protein.

Correction of abnormal CFTR is the process of enabling mutant CFTR to escape the cell's quality control machinery and be expressed in the apical plasma membrane where it would function correctly (Kreindler, 2010).

Early studies with non-pharmacological compounds such as glycerol (Sato et al., 1996) and dimethyl sulfoxide (Bebok et al., 1998) provided proof-of-principle that F508del-CFTR could be rescued from intracellular degradation to the same degree as low-temperature correction (Denning et al., 1992), though the exact mechanism of action was unknown.

The first identified pharmacological corrector of F508del-CFTR was 4-phenylbutyrate (Rubenstein et al., 1997; Rubenstein and Zeitlin, 1998), which was effective both *in vitro* and *in vivo*, although the *in vivo* effects were relatively small. It seems to act as a transcriptional activator through inhibition of histone deacetylases (Hutt et al., 2010).

Curcumin, a component of the Indian spice turmeric (*Curcuma longa*), is a SERCA [sarco (endo)plasmic reticulum calcium ATPase] pump inhibitor, for which different mechanisms of action have been proposed. The initial publication of the results of Curcumin-treated CF mice (Egan et al., 2004) led to high expectations. In this study, the electrophysiological defect was corrected in F508del homozygous mice treated with oral curcumin, but it has also been observed that some strains do not respond to curcumin treatment [e.g., (Grubb et al., 2006)]. In order to improve the bioavailability of curcumin, which is low and could vary across strains, in one study (Cartiera et al., 2010) poly lactic-*co*-glycolic acid (PLGA; a widely used biodegradable polymer) nanoparticles encapsulating curcumin were used to treat two different CF mouse strains. The nasal potential difference (NPD) data from this study suggest that oral administration of PLGA nanoparticles encapsulating curcumin enhances the

effects of curcumin therapy in CF mice, as compared to delivery of nonencapsulated curcumin.

Another recent study demonstrated that coexpression of F508del/N-half and C-half CFTR in the presence of correctors VX-325 and 2-(5-chloro-2-methoxyphenylamino)-4'-methyl-[4,5'bithiazolyl]-phenyl methanone (corr-4a) restores interactions between the two halves of F508del-CFTR, thus enhancing maturation of the mutant protein (Loo et al., 2009). It has also been reported that corr-4a significantly enhanced the protein stability of low-temperature-rescued F508del-CFTR for up to 12 hours at 37ºC in airway epithelial cells, although maximal function of the channel was not obtained (Jurkuvenaite et al., 2010).

Other drugs are currently being investigated to determine their efficacy as F508del-CFTR correctors (http://www.cff.org/research/DrugDevelopmentPipeline/).

F508del-CFTR has also a channel problem, and one way of overcoming this problem of decreased channel activity is to treat CF patients with a CFTR "potentiator" (Ameen et al., 2007). CFTR potentiators are molecules that have little or no impact upon the trafficking defects of mutant CFTR, but improve its channel gating characteristics, namely by increasing the open probability (*Po*) of mutant channels (Pedemonte et al., 2005b; Van Goor et al., 2006; Verkman et al., 2006).

The first recognized potentiator of CFTR was 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor that potentiated the cAMP-stimulated Cl^- currents in *Xenopus* oocytes injected with either F508del- or G551D-CFTR (Drumm et al., 1991).

Genistein, a flavonoid and tyrosine kinase inhibitor, activates G551D-CFTR channels (Illek et al., 1999) — which are present in the plasma membrane, but are inactive — and F508del-CFTR channels (Hwang et al., 1997; Schmidt et al., 2008). Therefore, genistein might be useful in individuals with the G551D mutation, and might enhance F508del-CFTR-mediated Cl^- secretion in patients who also receive a corrector that increases the membrane expression of F508del-CFTR (Guggino and Stanton, 2006).

Using a fluorescence-based high-throughput screen, Pedemonte and colleagues (Pedemonte et al., 2005a; Pedemonte et al., 2005b) identified sulfonamides as potentiators of F508del and phenylglycines as potentiators of F508del, G551D, and other mutant CFTR.

Another high-throughput approach led to the identification of 4-methyl-2-(5-phenyl-1H-pyrazol-3-yl)phenol (VRT-532) (Van Goor *et al.*, 2006), which has also been shown to act as a corrector (Wang et al., 2006), and VX-770, which increases

the amount of time that wt-, G551D- and F508del-CFTR channels are open (Van Goor et al., 2009).

It is unlikely that potentiators alone will yield an effective therapy for patients bearing the F508del mutation, since potentiators rely on being able to increase the activity of channels that are already present in the plasma membrane (Ameen et al., 2007). Thus, a combination of drugs acting on both channel activity (potentiators) and protein folding (correctors) will probably need to be utilized (Ameen et al., 2007; Proesmans et al., 2008). Interestingly, in a very recent study Mills and colleagues (Mills et al., 2010) have designed a hybrid molecule containing potentiator and corrector fragments linked through an ester bond. Cleavage of the hybrid molecule by intestinal enzymes under physiological conditions produced active potentiator and corrector fragments, providing proof-of-concept for small-molecule potentiator-corrector hybrids as a single drug therapy for CF caused by the F508del mutation.

Although all the above mentioned efforts are commendable, synthesis of a drug that can treat all CF cases would be difficult, if not impossible, given the *CFTR* mutational variability already pointed out. Thus, being non mutation-specific, gene therapy emerges as what could be called the perfect therapy for CF, since it could encompass all disease cases.

II. GENE THERAPY AND HUMAN ARTIFICIAL CHROMOSOMES (HACS)

II.1 GENE THERAPY FOR CF

Gene therapy can be defined as the introduction of an exogenous gene into a recipient cell to achieve a therapeutic benefit (Basu and Willard, 2005; Basu and Willard, 2006).

Cystic Fibrosis (CF) is an autosomal recessive disorder, which implies that a single copy of the wild-type *CFTR* gene is sufficient for normal function. Hence, CF became one of the first targets for gene therapy since apparently it is sufficient to supply the affected cells with a gene that expresses the CFTR protein. The therapeutic gene should be expressed in all epithelial cell types normally showing expression, preferably at levels comparable to the endogenous gene. It was shown that delivery of *CFTR* to as few as 6-10% of human CF airway epithelial cells *in vitro* could restore normal levels of chloride ion transport to levels comparable to those measured in non-CF cells, and as little as 8% of normal transcripts seem to preserve normal lung function (Chu et al., 1992). However, these experiments were performed

in homogeneous epithelial cell types that over-expressed CFTR using a retro-viral based vector and were polarized but undifferentiated. Thus, the cells analyzed did not represent the morphological characteristics of the human ciliated airway epithelium *in vivo* (Johnson et al., 1996). More recently, using a novel, recombinant virus vector (human parainfluenza virus, PIV) targeting ciliated cells and an *in vitro* model of human CF ciliated surface airway epithelium (CF HAE), it has been shown that restoration of airway surface liquid volume and mucus transport to CF HAE requires CFTR expression in ~25% of surface epithelial cells or approximately 30% of ciliated cells (Zhang et al., 2009). Although *in vivo* experimentation in appropriate models is required to confirm these data, the authors predict that a future goal for corrective gene transfer to the CF human airways *in vivo* would attempt to target at least 25% of surface epithelial cells to achieve mucus transport rates comparable to those in non-CF airways.

Moreover, expression should be stable and persistent, to avoid repeated re-administration of the therapeutic gene. The *CFTR* gene shows spatiotemporal expression regulation. Thus, long-term stability and regulated expression in a tissue-specific manner requires not only the promoter and coding portions of the *CFTR* gene but also regulatory and functional chromosomal elements such as enhancers and silencers associated with DNase I hypersensitive sites (DHS).

The airway epithelium is the most important target, as lung disease contributes mainly to morbidity and mortality in CF patients. The airway epithelium is a highly complex, multifunctional tissue. It lines the tubular structure of the airways from the nasal cavity, via the trachea into the intricately branched structure of the bronchial tree and consists of several epithelial cell types. These include, among others, mucus secreting goblet cells that produce a protective coating and ciliated cells that are involved in clearance of bacterial pathogens and other microscopic particles (Klink et al., 2004). However, the airway epithelial cell surface has innate barriers that seriously impair entry of therapeutic vectors, both viral and nonviral. These barriers have a very important function, given the masses of foreign DNA entering the lung during a lifetime, for example in the form of pollen, which might cause expression of birch genes, in the case of efficient gene transfer. The most important of these barriers is probably that birch genes are not stable in humans. In addition, the mucociliary clearance system and the glycocalyx (Pickles, 2004), a dense mucus-like mixture of carbohydrate, glycoproteins, and polysaccharides residing on the luminal surface of the epithelium of the airways (Kolb et al., 2006) efficiently prevent an accumulation of exogenous material. In the case of CF patients, the thick, sticky

sputum, consisting of inflammatory cells, cell debris, mucus and DNA, which is not effectively cleared out, forms an additional obstacle to vector uptake.

Given that CF, at least in the early stages, presents as a disease of the small airways, airway epithelial cells are likely to be important and considered by many to be the appropriate target cell for CF gene therapy (Griesenbach and Alton, 2009).

The long-term aims of gene therapy studies are the development of vectors as tools for studying genome and chromosome function and for delivering genes into cells for therapeutic applications (Grimes and Monaco, 2005).

II.2 GENE DELIVERY SYSTEMS (GDS)

An ideal gene-transfer vehicle would be a high-capacity, non-integrating vector that is capable of autonomous replication and maintenance in the host cell and capable of long-term regulated gene expression (Basu and Willard, 2005).

A gene delivery system (GDS) generally consists of a polynucleotide, encoding the therapeutic gene, and a carrier (Klink et al., 2004). The carrier must condense the polynucleotide, protecting it from mechanical stress and enzymatic attack. Furthermore, the carrier should facilitate transport of the therapeutic gene from the extracellular space into the cell and finally the nuclear compartment, where transcription can take place. In nature, specialists in gene delivery have evolved, namely viruses, which consist of a polynucleotide genome packaged in a protein structure called a capsid. The capsid proteins are well adapted to allow efficient binding of the virus to receptor proteins in target cells, facilitate intracellular transport and subsequent import of the viral genome into the nucleus. By replacing dispensable portions of the viral genome with a therapeutic gene, a recombinant virus can be created, which can be used as a natural GDS.

Nonviral gene delivery systems rely on cellular uptake mechanisms. Polycations complexed to DNA result in positively charged polyplexes that interact electrostatically with negatively charged proteoglycans of the plasma membrane, followed by endocytosis (Erbacher et al., 1999). To enhance specificity of cell uptake, attempts are made to shield the cationic aspect of the complexes with polyethylene glycol (PEG) moieties (Ogris et al., 1999; Rudolph et al., 2002; van Steenis et al., 2003).

Human artificial chromosomes (HACs) packed into cationic polymers are one such nonviral GDS. The primary aim of using HACs in gene therapy is to directly transfer HAC DNA to the target cells, where HACs form. Thus, therapeutic DNA can

be produced in high quantity and quality. In addition, several groups have attempted to isolate HACs once formed in cultured cells in a quantity sufficient to treat the large number of target cells of a patient. These latter HACs must be transferred out of the cell line in which they were generated and into therapeutically relevant cells for use in gene therapy applications (Irvine et al., 2005).

To date, studies have focused on generating HACs in HT1080 cells which allow efficient HAC formation and analysis due to the pseudostable karyotype of this telomerase positive lung sarcoma cell line (Ebersole et al., 2000; Grimes et al., 2001; Ikeno et al., 2002; Laner et al., 2004; Kaname et al., 2005; Kotzamanis et al., 2005; Laner et al., 2005; Suzuki et al., 2006).

There are several different methods for the transfer of HACs between cell types but, with the exception of microcell-mediated chromosome transfer (MMCT) (Kakeda et al., 2005), methods for transferring HACs into human somatic cells require an initial purification step to isolate HACs from other human chromosomes and chromosome fragments. Flow cytometric sorting is the most efficient method of isolating populations of purified human chromosomes with their associated chromatin modifications intact, which is possible to some degree of purity for chromosomes of a minimal size of around 60 Mb, similar to human chromosome 21 (deJong et al., 1999). Transfection or microinjection could also be used to transfer HACs into human somatic cells, should the purification of functional populations of HACs be achieved (Irvine et al., 2005).

Another approach under development for a direct delivery of large DNA molecules is the bacterial invasin system (Grillot-Courvalin et al., 1998; Narayanan and Warburton, 2003; Laner et al., 2005). 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).

After entering the cell and having reached the perinuclear region, the heterologous DNA must enter the nucleus for transcription. In principle, there are two ways for viral and nonviral vectors to deliver their genetic material into the nucleus. First, the vector resides in the cytosol until the nuclear envelope is disassembled during mitosis. The vector genome is then included into the newly assembling nuclei of the daughter cells. Alternatively, the genomic material can be delivered by active transport through the envelope of the interphase nucleus (Whittaker, 2003; Klink et al., 2004).

II.2.1 VIRAL VECTORS

GDS have been derived from various viruses. Adenovirus [Ad; (St George, 2003; Mueller and Flotte, 2008)], adeno-associated virus [AAV; (Zhang et al., 1998; Ostedgaard et al., 2002; Buning et al., 2003; Ostedgaard et al., 2005; Fischer et al., 2007; Daya and Berns, 2008; Li et al., 2009)], Sendai virus [SeV; (Bitzer et al., 2003a; Bitzer et al., 2003b; Griesenbach et al., 2005; Ferrari et al., 2007; Rakonczay, Jr. et al., 2008)], herpes simplex virus [HSV; (Wade-Martins et al., 2001; Shah et al., 2003; Wade-Martins et al., 2003; Inoue et al., 2004; Moralli et al., 2006)], and Epstein-Barr virus [EBV;(Wade-Martins et al., 2000; Mazda, 2002; Stoll and Calos, 2002; Black and Vos, 2002; Magin-Lachmann et al., 2003; Conese et al., 2004; Kotzamanis et al., 2009)] based-vectors, among others, are investigated for gene transfer. Viruses' innate ability to infect the airways made them a natural initial choice for CF gene therapy, and many of the earlier studies were performed with viral vectors derived from adenovirus.

In general, the main advantage of viral vectors is the high transduction efficiency *in vivo*, compared to current synthetic systems. Further, the use of integrating viral vectors such as Adeno-associated virus (AAV) and lentiviral vectors would allow stable expression in the targeted cell pool (Verma and Somia, 1997).

On the down side, viral-based gene therapy poses serious safety concerns like generation of an acute host defense response (Crystal et al., 1994; Zhang et al., 2001; Harvey et al., 2002), and a cytotoxic T-lymphocyte response which can develop against cells that produce viral antigens encoded by the vector (St George, 2003). Another disadvantage of virally based non-integrating vectors is their failure to segregate in a regulated manner (Basu and Willard, 2005). In the case of integrating vectors, insertional mutagenesis is a major concern, as for oncoretroviral vectors (Hacein-Bey-Abina et al., 2003; Fischer et al., 2004; Conese et al., 2007).

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 be expressed only for a limited period of time (Porteous et al., 1997). One of the reasons may be that viral promoters are known to be switched off through the action of cytokines (Paillard, 1997).

II.2.2 SYNTHETIC VECTOR SYSTEMS

A method for overcoming both the lack of packaging capacity of AAV vectors (which are associated with a low incidence of inflammation) and the immunogenicity of viral vectors in general for gene transfer is to make synthetic GDS. These consist generally of DNA encoding the therapeutic gene, combined with a carrier that mimics the properties of viral capsids, to package and deliver therapeutic genes. Carriers include cationic lipids complexed with DNA and compacted DNA nanoparticles (cationic polymers) and can potentially package any size of DNA. They condense the polynucleotide molecule, bind to cells, and help the vector to escape from the endosomal compartment, avoiding degradation (Klink et al., 2004; Kreindler, 2010).

Although many carriers have been used successfully *in vitro*, their *in vivo* use is less straightforward. Initial studies and clinical trials that targeted airway epithelia were performed with cationic liposomes, which were used as nonviral gene transfer vectors for treating CF. Cationic lipid-mediated DNA transfer to the nasal epithelium of CF patients were promising, but failed to demonstrate persistent correction of nasal potential difference (NPD) abnormalities (Caplen et al., 1995; Goddard et al., 1997; Mueller and Flotte, 2008; Kreindler, 2010). Nonetheless, proof of principle was established that marker genes and *CFTR* could indeed be expressed in this way (Caplen et al., 1995). One of the most successful studies on CF patients was conducted by Alton et al. where cationic liposomes containing the CF gene were delivered by a nasal perfusion to CF patients (Alton et al., 1999). There was, however, an innate inflammatory response from the CF patients to inhalation of the complexes.

Liposomes are attractive, as by themselves they appear to be nonimmunogenic. However, when used in gene therapy (i.e., with plasmid inside), they generate a significant immune response, probably triggered by the CpG sequences in the vector, and while repeated delivery is feasible, the overall efficiency of transgene expression is low (Kay et al., 1997; Sallenave et al., 1997). Thus, through extensive nucleotide optimization, the UK CF Gene Therapy Consortium has generated a CFTR expression plasmid, which is completely depleted of all CpG sequences (Hyde et al., 2008).

The other class of synthetic nonviral vectors used for gene therapy in CF is cationic polymers such as poly-L-lysine (Poly-K), polyethylenimine (PEI), and polyamidoamine dendrimers. Like cationic liposomes, polymers are able to condense DNA into small nuclease-resistant particles. Due to their net positive charge, cationic polymers can bind to cells via electrostatic interactions with the negatively charged

membrane (Mueller and Flotte, 2008). One such polymer is a polyethyleneglycol (PEG)-substituted 30-mer polylysine peptides (polymer of 30 lysines) that complexes with DNA to form essentially charge-neutral DNA nanoparticles. A double-blind, placebo controlled dose escalation trial of DNA nanoparticles to the nasal epithelium of CF subjects demonstrated gene transfer and transient correction of NPD abnormalities without evidence of local or systemic inflammation (Konstan et al., 2004).

II.2.3 CHROMOSOMAL VECTORS

Drawbacks in the use of classical gene transfer vectors include i) immune response to viral proteins or ii) to unmethylated CpG motifs contained in bacterially-derived vector DNA, and iii) shut-off of viral promoters (Conese et al., 2007).

Nonviral vectors have attracted a large amount of attention in recent years because of lack of specific immune responses, endogenous virus recombination or oncogenic effects, as can occur with viral gene transfer agents (Thomas et al., 2003).

There are two essential advantages of chromosome-based vector systems over most conventional vectors for gene delivery. First, the transferred DNA can be stably maintained without the risks associated with insertion, and second, large DNA segments encompassing genes and their regulatory elements can be introduced, leading to more reliable and physiological transgene expression, more closely resembling that of the normal gene. Furthermore, chromosomal vectors comprised solely of human DNA should not be immunogenic (Vassaux, 1999; Saffery and Choo, 2002; Grimes and Monaco, 2005).

The concept of artificial chromosomes was introduced nearly thirty years ago in *S. cerevisiae* (Murray and Szostak, 1983), referring to the construction of a fully functional chromosome from its component parts.

Stripped to its essentials, a typical chromosome is composed of centromeres, telomeres, origins of replication, and genes. Telomeres are responsible for capping and protecting the linear ends of chromosomes from degradation and recombination. Perhaps most important from the standpoint of long-term stability, the centromere is the *cis*-acting chromosomal structure responsible for mediating the establishment of the kinetochore, a trilaminar protein/DNA complex responsible for establishing attachments to and movements of the chromosome along the mitotic spindle apparatus (Wade-Martins et al., 1999 ;Lipps et al., 2003). Origins of replication, although unidentified on the sequence level, [reviewed in (Gilbert, 2001)] do not

seem to pose a problem since in the presence of centromeric DNA large pieces of DNA (few tens of kb) are generally competent for replication (Conese et al., 2004).

There have been two main approaches used for generation of chromosome-based vectors (Figure I.6). These involve either 1) the use of telomeric DNA to sequentially truncate human chromosomes and generate smaller derivative minichromosomes ("top-down" approach), and 2) a fundamentally different strategy, which involves a "bottom-up" approach in which cloned chromosomal elements, including alpha-satellite DNA, telomeric DNA, and genomic DNA, are preassembled into a defined artificial chromosome vector or are assembled spontaneously by the host cell through a combination of nonhomologous recombination and DNA repair mechanisms (Harrington et al., 1997; Ikeno et al., 1998; Grimes and Monaco, 2005).

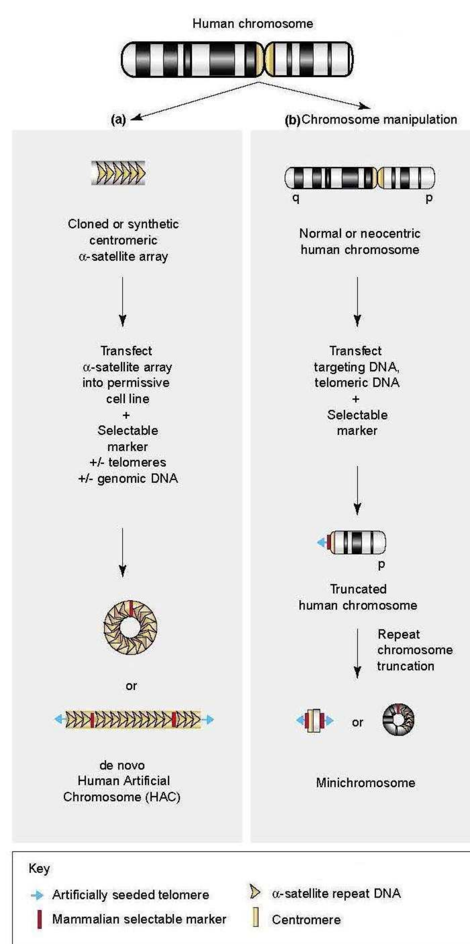

Figure I.6 Strategies for the construction of HACs. **(a)** The "bottom up" or *de novo* approach involves the transfection of deproteinated α-satellite DNA arrays and a selectable marker gene, with or without telomeric DNA and human genomic DNA, into a permissive cell line. **(b)** HAC formation by the "top down" approach involves sequential rounds of random and/or targeted truncation of human chromosomes through interstitial insertion of terminal telomeric DNA sequences. The long arm of the chromosome is denoted by q and the short arm by p. [Adapted from (Irvine et al., 2005)].

The molecules obtained from the *de novo* (bottom-up) and top-down approach share some important similarities. They are mitotically stable and maintained at low copy numbers and are 1 – 10 Mb in size and composed of alphoid DNA confirming the primary role of the centromeric DNA in chromosome maintenance (Conese et al., 2004). Beyond these similarities, *de novo* chromosomes, with the exception of a few linear examples, are suspected to be circular, even when produced with linear constructs. The fact that they are larger than the input DNA is probably due to a multimerization process that produces an undefined structure (Conese et al., 2004).

Conversely, the minichromosomes (MCs) maintain the linear structure of the natural chromosomes from which they derive and have functional telomeres. Moreover, it has recently been shown that *de novo* chromosomes exhibit a higher frequency of segregation errors with respect to natural chromosomes. Two common types of errors have been observed: nondisjunction and anaphase lag. The frequency of these defects is statistically different between natural and artificial chromosomes suggesting that the latter may be less stable (Rudd et al., 2003). Although MCs have not been tested for segregation errors, analysis of their mitotic stability revealed a decreased stability of small MCs with respect to larger molecules of the same origin, suggesting the requirements of an optimal size for full chromosome function.

There remain significant technical challenges that must be overcome before vectors carrying the therapeutic gene will be ready for gene transfer applications, the most important being determination of the optimal structure and size of the gene of interest and delivering it efficiently to target cells.

II.2.3.1 YACs

Because yeast artificial chromosomes (YACs) are maintained as linear molecules, they are prone to shearing by mechanical stresses. YACs can also be difficult to purify from similarly sized endogenous yeast chromosomes as they are typically separated from endogenous chromosomes by pulse field gel electrophoresis (PFGE) of agarose-embedded yeast cells. Furthermore, YAC libraries have a high rate of insert chimerism, *i.e.* cloning of non-contiguous sequences in a single clone, and insert rearrangements/deletions, a result of the active homologous recombination machinery of the yeast host (Neil et al., 1990; Kouprina et al., 1994; Larionov et al., 1994).

YACs containing the human *CFTR* locus (Chr 7q31) were isolated shortly after the identification of the *CFTR* gene (Anand et al., 1991). However, yeast elements are not stable in higher eukaryotes (Klink et al., 2004).

II.2.3.2 BACS AND PACS

Because of the inherently unstable nature of YACs, alternative cloning systems able to propagate large genomic fragments were sought. The first YAC alternative developed was the P1 phage cloning system (Sternberg, 1990; Glover and Hames, 1995). The P1 cloning system has many advantages over YACs. The bacterial host is recA$^-$, and therefore does not readily rearrange the genomic insert by homologous recombination (Sternberg, 1990). Additionally, the P1 clones are maintained as a single copy (Sternberg, 1990). Moreover, the supercoiled, circular nature of P1 clones makes them less susceptible to mechanical shearing. While P1 phage clones are much more stable than YACs, the size of their genomic insert is more limited. The P1 phage head can accommodate up to only 110 kb of DNA (Glover and Hames, 1995; Giraldo and Montoliu, 2001).

The bacterial artificial chromosome (BAC) cloning system is based on the *Escherichia coli* (*E. coli*) fertility (F) factor (Shizuya et al., 1992). The maximum observed mammalian genomic insert size carried by BACs is around 300 kb (Shizuya et al., 1992; Kim et al., 1996). As with the P1 phage system, BACs are circular molecules resistant to mechanical shearing and can be isolated using conventional plasmid purification protocols (Yang et al., 1997). Just as with P1 phage clones, BACs are maintained in a recA$^-$ host and are much more stable than YACs (Heaney and Bronson, 2006).

Phage artificial chromosomes (PACs) were developed to combine the unique features of BACs and P1 phage (Ioannou et al., 1994; Frengen et al., 2000). The PAC is a modified version of the P1 phage plasmid that, after ligation to partially digested genomic DNA, is electroporated as a circular molecule into the bacterial host, rather than being packaged into bacteriophage particles (Strong et al., 1997). By averting the packaging step, PAC clones can be maintained as genomic inserts of the same size as BACs (Ioannou et al., 1994). Additionally, some PACs contain the P1 lytic replicon, which can be activated by IPTG to increase PAC copy number prior to DNA purification, resulting in increased DNA yield (Ioannou et al., 1994).

The stability and relatively large insert capacity of BAC and PAC clones have made them ideal for high-resolution physical mapping. As such, BACs and PACs

were the cloning system of choice for constructing physical maps of the publicly-funded human and mouse genome sequencing projects. With the current sequence information available for most BAC and PAC clones, these artificial chromosomes have become a popular resource for artificial chromosome-based transgenes (Heaney and Bronson, 2006).

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, the locus of interest often needs to be assembled from two or more different vectors according to the contig maps (as was the case in the present work). This may be a difficult and time consuming step, although methods based on Red gene-mediated homologous recombination supplied by defective lambda prophage, have been developed to retrofit and modify PAC/BAC vectors (Lee et al., 2001;Kotzamanis et al., 2005). The generation of BAC vectors containing the entire cystic fibrosis transmembrane regulator (*CFTR*) and factor VIII genes assembled from overlapping BACs using homologous recombination and their expression have been reported (Perez-Luz et al., 2007; Kotzamanis et al., 2009).

A PAC has been engineered to contain a large region of the *CFTR* gene (140 kb) including its natural promoter, fused to a synthetic exon encoding eGFP. Expression from the CFTR promoter by RT-PCR, splicing of all 10 exons, and correct translation of the expected CFTR-eGFP fusion protein as well as reliable detection of a stable copy has been demonstrated in mammalian cells (Laner et al., 2005). We describe here the assembly of a CF PAC from previously characterized PAC resources (Ramalho et al., 2004) carrying a 225 kb genomic insert including virtually the whole *CFTR* gene (with the exception of two deletions in introns 9 and 10) and flanking genomic regions.

II.2.4 HUMAN ARTIFICIAL CHROMOSOMES (HACS)

Normal mammalian chromosomes are linear and have three essential functional elements: telomeres (for protection and maintenance of chromosome ends), replication origins (for DNA duplication), and a centromere (for equal segregation at cell division; for more details about the centromere, see section II.2.4.1 below).

Human artificial chromosomes (HACs) are autonomous molecules that can function and segregate as normal chromosomes in human cells (Monaco and Moralli, 2006). HACs may be either linear or circular molecules with clearly defined centromeric (alpha-satellite DNA), replication origin, and, where relevant, telomeric elements, all of which typically are cloned into a BAC, YAC, or PAC vector backbone (Ebersole et al., 2000; Schueler et al., 2001; Grimes et al., 2002; Mejia et al., 2002; Ohzeki et al., 2002; Kouprina et al., 2003; Rudd et al., 2003; Laner et al., 2004).

The purpose of engineering and manipulating HACs for gene transfer studies/applications is twofold. First, most mammalian genes are large, certainly larger than can be packaged as a genomic fragment into viral vector capsids (Somia and Verma, 2000). Second, the properly controlled expression of mammalian genes often depends on as yet poorly defined genomic regulatory elements (associated with DHS) that can reside a substantial distance from the coding sequences themselves (Li et al., 2002). Thus, HACs contain all the functional elements for long-term stability within cells offering the possibility of long-term gene expression in human cells and the development of future somatic gene therapy. HACs are validated by confirming their *de novo* composition and high mitotic stability when grown for long periods in culture without selection (Grimes and Monaco, 2005).

Two approaches can be used to load the therapeutic gene into an HAC: i) site specific recombination into a preformed HAC (*in vivo* recombination), and ii) assembly of a *de novo* chromosome with the gene of interest and centromere sequences (*in vitro* recombination) (Conese et al., 2007). Systems that have been developed for the *in vivo* recombination approach include site-specific recombination at *lox*P sites catalyzed by Cre recombinase from the *E. coli* phage P1 (Mejia and Larin, 2000), Red-mediated recombination enzymes from phage lambda (Kotzamanis and Huxley, 2004; Kotzamanis et al., 2005), and bacterial transposition (Basu et al., 2005b); methods that have been used for the *in vitro* recombination approach include conventional subcloning with restriction digestion and ligation (Mejia and Monaco, 1997), (this work), and in gel site specific recombination (Schindelhauer and Cooke, 1997).

In the present work an attempt was made at assembling a *de novo* HAC by an *in vitro* recombination approach using restriction digestion and ligation of a construct carrying the therapeutic gene (*CFTR*) and adjacent genomic sequences, and a PAC-based vector containing centromeric DNA sequences. An *in vitro* recombination approach was chosen for the assembly of a *de novo* HAC because these are more reproducible and less rearrangement prone than *in vivo* recombination approaches.

As already implied above, the main purpose for constructing HACs is the delivery of therapeutic genes into patients' cells. As a proof-of-principle, several studies have demonstrated the efficacy of *de novo* HACs for delivery and expression of large human transgenes in human cell lines. Mitotically stable HACs expressing either the human hypoxanthine guanine phosphoribosyltransferase (*HPRT*) (Mejia and Larin, 2000; Grimes et al., 2001) or the guanosine triphosphate cyclohydrolase (*GCH1*) (Ikeno et al., 2002) genes were constructed either using a co-transfection strategy or from pre-assembled vectors.

Although progress has been made in the development of gene expressing HACs, we still have a long way ahead of us before being able to control the whole process of HAC construction. Currently, the process of *de novo* HAC assembly within cells is poorly understood and has been achieved in a limited number of human cell lines, predominantly the fibrosarcoma cell line HT1080 (Irvine et al., 2005). A stable HAC was also formed in primary pig cells and Hiroshi Masumoto and colleagues formed a HAC in murine cells (Dirk Schindelhauer, personal communication).

Additionally, the *de novo* HACs reported to date typically are formed by an uncontrolled concatemerization of the starting vector, to form mega-base-sized derivatives (Harrington et al., 1997; Ikeno et al., 1998; Mejia et al., 2001; Schueler et al., 2001; Grimes et al., 2002; Ohzeki et al., 2002; Rudd et al., 2003; Basu et al., 2005a; Basu et al., 2005b).

The centromere and the kinetochore which is assembled upon it are two cell structures essential for assuring episomal maintenance and transmission of HACs to daughter cells during cell division. To date, several groups have demonstrated that the centromere-specific histone variant, CENP-A (centromere protein A), is deposited on *de novo* artificial chromosomes, consistent with its central role in kinetochore formation (Masumoto et al., 1998; Grimes et al., 2001; Grimes et al., 2002; Ikeno et al., 2002; Ohzeki et al., 2002). However, the efficiency of *de novo* HAC formation and stability depends on the presence of another protein, CENP-B, which binds to a sequence called CENP-B box (Ohzeki et al., 2002; Basu et al., 2005b) and, to some extent, on the chromosome origin of the alphoid template and the longer length of the alphoid array (>100 kb) (Kaname et al., 2005). Given the role played by both the centromere and the kinetochore in the formation of an independently replicated, persistent HAC, their structure and composition will be further described below.

II.2.4.1 THE CENTROMERE

The centromere is the key functional component of an autonomous, mitotically stable gene therapy vector since it directs the equal segregation of genomic material into daughter cells during mitosis. It is a complex proteinaceous structure which appears as the cytologically visible primary constriction on mitotic chromosomes of higher eukaryotes.

Human centromeres are composed of 0.2-7 megabases of a 171 bp sequence (alpha-satellite motif) repeated in tandem head-to-tail arrangement (Maio, 1971; Choo et al., 1991). They play multiple roles in the control of segregation at cell division, including: assembly of a kinetochore and spindle attachment, maintenance of sister chromatid cohesion until anaphase onset, and movement of chromosomes to opposite poles (Cleveland et al., 2003; Vagnarelli et al., 2008).

The principal class of centromeric DNA in higher eukaryotes is the above mentioned alpha-satellite DNA. Alpha-satellite can be subdivided into two types (Ikeno et al., 1994; Alexandrov et al., 2001). Type 1 forms regular higher order repeat arrays, is associated with centromere function and contains a 17 bp motif known as the CENP-B box which represents the binding site for CENP-B (Earnshaw and Rothfield, 1985; Earnshaw et al., 1987; Masumoto et al., 1989). Type 2 is monomeric alpha-satellite and lacks a regular higher-order organization. This type of alpha-satellite DNA usually flanks the type 1 array and is often interrupted by LINE and SINE sequences (Prades et al., 1996).

The importance of alpha-satellite DNA is highlighted by the fact that it is the only element capable of independently nucleating centromere formation *de novo* and members of certain human alpha satellite DNA families were found to form active centromeres *de novo* when transfected into mammalian cells (Harrington et al., 1997; Saffery et al., 2001). The mechanism by which cloned alpha-satellite DNA serves as a template for the recruitment of centromere-specific proteins and the assembly of a functional kinetochore remains to be elucidated (Basu and Willard, 2005).

Kinetochores are the large protein complexes that mediate segregation in higher eukaryotes. They interact on one side (inner kinetochore) with the chromosomes and on the other side (outer kinetochore) with the spindle microtubules. Thus, the kinetochore serves as the protein interface between the chromosomes and the spindle apparatus that drives chromosome segregation (Kanizay and Dawe, 2009). The centromeres serve as scaffolds for kinetochores, which are assembled immediately before and during the first steps of mitosis (Vagnarelli et al., 2008; Cheeseman and Desai, 2008). The kinetochore is composed

of a number of constitutive proteins, including CENP-A, as well as the centromeric chromatin-associated proteins CENP-B and -C, which form the foundation on which the kinetochore assembles.

It is well established that epigenetic factors (methylation, acetylation, and phosphorylation of amino acids), and not the DNA sequence, are responsible for centromere identity (Dalal, 2009). A key component of this epigenetic marking system is the centromere-specific CENP-A protein (Palmer et al., 1991). CENP-A is a member of a family of evolutionarily conserved, centromere-specific histone H3s that package centromeric DNA at the kinetochore into a specialized chromatin structure (Sullivan et al., 2001; Amor et al., 2004). Centromeres are identified by their interaction with CENP-A (Kanizay and Dawe, 2009).

Recent studies revealed that CENP-B, another component of the kinetochore, has a dual role, on the one hand recruiting CENP-A to the chromatin during *de novo* centromere formation, and on the other actively enhancing the H3K9Me3 (histone H3 tri-methylated at lysine 9) modification of chromatin containing chromosomally-integrated alpha-satellite DNA without stimulating CENP-A assembly (Okada et al., 2007).

OBJECTIVES

The high mutational variability affecting the *CFTR* gene and consequently the great phenotype variability of the CF disease is a major impairment to the design of a single therapy for CF using drugs. Gene therapy, being non mutation-specific, seems the perfect answer for the treatment of this common genetic disease. By restoring CFTR function through the delivery of a healthy copy of the *CFTR* gene to the affected cells, it would provide a cure regardless of the mutation involved. Unfortunately, gene therapy in general and for CF is not a straightforward strategy, as many variables have to be taken into consideration (such as, among others, the most appropriate vector to be used, the level of expression desired, gene silencing, a possible immune response, and reducing the need for administration of repeated doses), all of which make gene therapy a work in progress and not yet a reality. Despite all the work that has already been achieved in the field, there are still some steps to be carried out before a CF cure by gene therapy can become a reality. In this work we addressed CF gene therapy in a specific way, having as final aim the formation of a human artificial chromosome (HAC) expressing the *CFTR* gene under the control of the endogenous promoter that could be delivered to CF affected cells. In order to achieve this important step for CF gene therapy, the objectives of the present work were:

- To design and produce a construct containing the *CFTR* locus in an appropriate vector which would allow the formation of a CFTR HAC. And we obtained this by:
  - Assembling a tagged *CFTR* locus containing all 27 exons and flanking genomic regions in a single PAC, from characterized DNA preparations of resource PACs.

- To determine if the *CFTR* locus was expressed and HAC formation could occur in human pulmonary cells. In order to answer these questions, we:
  - Co-transfected human pulmonary cells with the *CFTR* construct and a centromere proficient alpha-satellite construct.

- To assemble a *de novo* HAC by ligating the *CFTR* construct and a PAC-based vector containing centromeric and telomeric DNA sequences plus a selectable marker and cloning it into *E. coli* cells.

CHAPTER II

RESULTS

CHAPTER II. RESULTS

1. SUMMARY

Classical gene therapy to cystic fibrosis has had limited success due to immune response against viral vectors and short-term expression of cDNA based transgenes. These limitations could be overcome by delivering the complete genomic *CFTR* gene on non-integrating human artificial chromosomes (HACs). Here, the reconstruction of the genomic *CFTR* locus into one P1-based artificial chromosome (PAC), CF225, is reported. CF225 is a midsized, non-selectable PAC (225.3 kb, -60.7 kb to +9.8 kb) ligated from two PACs with optimized codons and a silent *Xma*I restriction variant to discriminate transgene from endogenous expression. After co-transfection with telomerized, blasticidin-S selectable, centromere-proficient alpha satellite constructs into HT1080 cells, CF225 was not incorporated into a *de novo* HAC in 122 lines analyzed, but integrants expressed. Stability analyses suggest feasibility to pre-fabricate a large, tagged *CFTR* transgene that stably replicates in the proximity of a functional centromere. Although definite conclusions about HAC proficient construct configurations cannot be drawn at this stage, important transfer resources were generated and characterized, demonstrating promise of *de novo* HACs as potentially ideal gene-therapy vector systems.

2. INTRODUCTION

The primary transcript of the *CFTR* gene is ~189 kb long and comprises 27 exons (Rommens et al., 1989). The adjacent genes, *GASZ* and *CORTBP2*, show different nuclear localization in human cells, depending on their differently regulated expression (Zink et al., 2004; Sadoni et al., 2008), and therefore sequences of these genes are unlikely to belong to the chromatin domain that regulates *CFTR*. The distance between the genomic regions of the primary transcripts of the adjacent genes is 283 kb, suggesting that the size of the functional *CFTR* locus is between 189 and 283 kb. The gene order and exon structure around the *CFTR* locus is highly conserved in vertebrates (Sadoni et al., 2008), posing the question why this order was maintained over 500 M years in the absence of gene regulatory constraints. It is

presently not known if all intronic and extragenic sequences of the *CFTR* locus are required for normal gene expression or play a role in locus stability.

Since CF is a recessive disorder, a single copy of the normal *CFTR* gene is sufficient to achieve functional CFTR levels that avoid CF, making CF an attractive candidate disease for gene therapy. It is expected that low levels of stable expression of the *CFTR* gene inside defective cells, or correction of even a fraction of cells of the epithelium could alter pathological epithelial physiology, thus being of clinical benefit (Dorin et al., 1996; Ramalho et al., 2002).

Thus, successful gene therapy requires persistent tissue specific expression of the transgene, which should be optimally achieved by delivery of a complete locus of genomic DNA including native regulatory and promoter elements. To avoid random integration into the host chromosomes and allow stable inheritance, additional genetic elements are required. The most important of these elements is a functional centromere. Human artificial chromosomes (HACs) based either on centromeric alpha satellite DNA (i.e. long arrays of tandem repeats > 80 kb) as the only human component in a circular P1 phage-based artificial chromosome (PAC), or on linear, telomerized alpha-satellite DNA, faithfully replicate and segregate during mitosis for many cell divisions in the absence of selection (Ebersole et al., 2000; Grimes et al., 2001).

To achieve this goal for CF, in addition to the complete *CFTR* genomic locus (including the promoter and regulatory sequences in introns and outside of the primary transcript) vectors to be transfected into CFTR expressing epithelial cells should also carry a functional centromere and telomeres. Furthermore, correct splicing and expression of the transgene must also occur from the *de novo* formed HAC. The stability and relatively large insert capacity of P1 and F factor based artificial chromosomes (PACs/BACs) make them ideal for cloning large genomic sequences (Shizuya et al., 1992; Ioannou et al., 1994).

The *de novo* formation of HACs following transfer of naked DNA molecules is a poorly understood process of DNA assembly, concatemerization, and chromatinization, which leads to individual genetic entities (i.e. novel chromosomes) in the recipient cells. Some of the assembled structures from a co-transfection may contain all transferred sequences in a composition suitable to acquire the different chromatin regions that are required for the different functional domains of a HAC. Regulated gene expression requires open chromatin in suitable cell types. A specialized open chromatin domain and faithfully replicating portions of heterochromatin are required for centromere formation, ensuring attachment to the mitotic spindle and segregation (Nakano et al., 2008). Active centromeres are

marked by the histone H3 variant CENP-A, and need to be protected from adjacent gene expression. Co-transfections of large DNA sequences are usually inefficient, but in combination with intact DNA preparations can represent a workable strategy to characterize function of a cloned DNA fragment and determine suitability for the further pre-fabrication of a HAC construct containing all required sequences in a single molecule.

After the production of the genomic construct CGT21 (Figure II.1A) containing about one half of the *CFTR* gene locus and a tagged last exon, and the demonstration that it was stably propagated in lung sarcoma cells, where it was expressed and correctly spliced (Laner et al., 2005), the next logical step was to generate a tagged genomic *CFTR* construct carrying all 27 exons and flanking regulatory sequences for incorporation into a HAC.

We describe herein for the first time, the assembly of a tagged *CFTR* locus containing all 27 exons and most of the potential regulatory regions in a single PAC. Starting from stored, characterized DNA preparations of resource PACs containing *CFTR*, we ligated two PACs, CF1 and CF6, with a corrected exon 10/intron junction fragment and obtained a clone of interest, termed CF225 (Figure II.1A). This clone has the advantage of containing the optimized methionine (M) codon at the polymorphic M470V locus compared to the wt resource clones and a synthetic, silent *Xma*I restriction variant, which is suitable for discrimination of RT-PCR products of the transgene from the endogenous *CFTR* loci of any target cell.

Here, to achieve incorporation of the *CFTR* locus into HACs, we co-transfected the cloned *CFTR* loci with centromere proficient alpha-satellite constructs and analysed their expression.

3. RESULTS

3.1 CONSTRUCTION OF THE FUSION PAC CF225 FROM CHARACTERIZED RESOURCE CLONES

Here, we aimed at constructing a tagged version of the entire genomic *CFTR* gene cloned in a P1-phage suitable for large scale, high quality DNA preparation. In addition to the previously described construct CGT21 carrying a tagged half locus (Laner et al., 2005) we constructed PAC CF225 carrying the human *CFTR* gene with all exons and introns plus regulatory sequences by ligating two PACs, CF1-Met (i.e. with M at the M470V locus) and CF6, each containing roughly half of the *CFTR* gene

and flanking regions (Figure II.1). PAC CF1-Met carries an insert running from -60.7 kb upstream from the start of translation in exon 1 to intron 10, and PAC CF6 carries an insert running from intron 10 to +9.8 kb (relative to the end of translation) of downstream DNA. Due to the choice of the resource PACs, both introns 9 and 10 are substantially shortened by 5.1 and 27.1 kb, respectively (Figure II.1A). As a

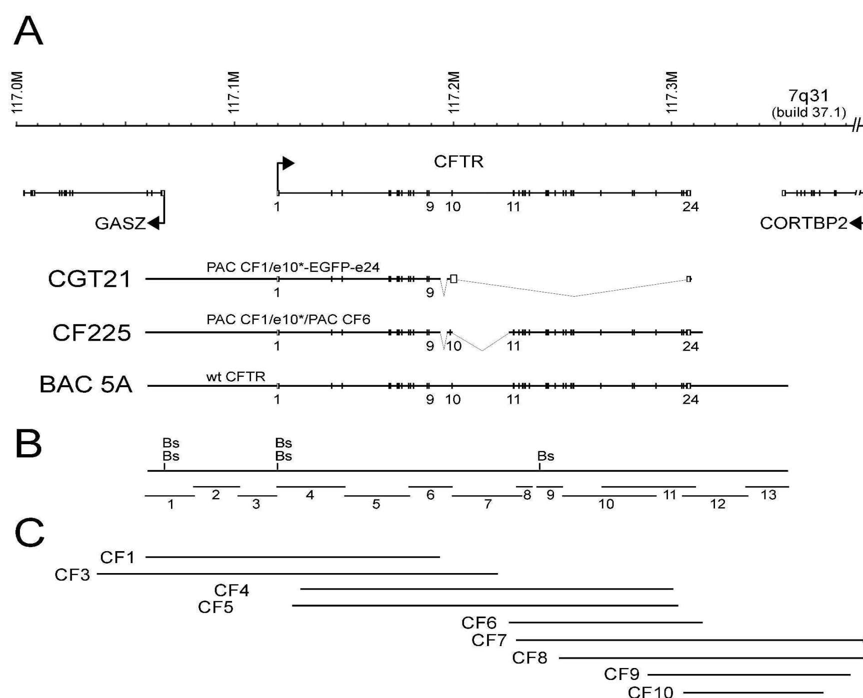

Figure II.1 Schematic of the *CFTR* locus, constructs and resource clones drawn to scale. **(A)** The size bar shows the position on the chromosome 7 sequence, according to build 37.1 of the hg sequence. The primary transcripts of the *CFTR* gene and the differently regulated, adjacent genes *GASZ* and *CORTBP2* are shown with their intron and exon structure and transcript direction. Three construct inserts (bold lines) are shown and the *CFTR* exon/intron content and adjacent regions are given. CGT21 contains one half of the *CFTR* locus and is based on PAC CF1, which was joined to an engineered exon 10 (open box) consisting of partial exon 10, EGFP-coding, and partial exon 24 sequences, including the stop codon of *CFTR* and 0.5 kb 3' region. Regions not covered by the construct are joined by thin lines. Intron 9 of construct CGT21 lacks 5.1 kb, which was known to not impair expression and correct splicing (Laner et al., 2005). Construct CF225 is based on a modified PAC CF1 (CF1-Met required during construction of CGT21) and CF6, which were joined with an engineered exon 10 sequence including splice junctions. Intron 9 lacks 5.1 kb and intron 10 lacks 27.1 kb regions not present in the chosen resource PACs. Both, CGT21 and CF225 contain an optimized sequence in exon 10 (asterisk) encoding the 470M variant and contain the silent *Xma*I variant introduced for the unambiguous transcript detection compared to endogenous human *CFTR* genes. Construct 5A represents the entire wt *CFTR* locus and extends into adjacent genes, suggesting presence of all regulatory regions of the *CFTR* related chromatin domain. **(B)** Shown are 13 long range PCR reactions covering the entire *CFTR* locus, except a 3 kb gap between reactions 8 and 9 in intron 14. LR-PCR based fine mapping was used to compare the structure of the *CFTR* locus cloned in the different PAC resources (Table 1). All products were obtained from the different sources. For comparison, positions of *Bss*HII (Bs) restriction sites are given. **(C)** Characterized resource PACs with published exon/intron junction sequences (Ramalho et al., 2004) covering the *CFTR* locus.

consequence, the described DNase I hypersensitive sites (DHS) in intron 10 (McCarthy and Harris, 2005) are excluded from locus CF225.

The nine PAC clones, CF1-CF10 (Figure II.1C) covering the human *CFTR* gene locus (a resource from the human genome project) were analysed here for exon content by using PCR. Since approximately 1 in 25 caucasians carries a *CFTR* mutation, and functionally relevant polymorphisms exist, all exons and splice junctions of the resource PACs were sequenced prior to construction. PCR primers and sequencing data are summarized online (Ramalho et al., 2004) and sequences are available under EMBL/GenBank accession numbers AJ574939-AJ575055. PACs CF1-CF5 were found to contain the common splice variant $(TG)_{11}T_7$ described to result in a small proportion of an alternatively spliced product lacking exon 9 (Cuppens et al., 1998; Ramalho et al., 2002), but mostly producing a normal transcript. Exon 10 of PACs CF3-CF5 was found to encode valine at amino acid position 470, which was exchanged to methionine during construction of CF1-Met PAC, giving rise to a 1.7 fold higher chloride conductance activity (Cuppens et al., 1998). Construction of the CF1-Met PAC required a number of engineering steps. In short, we used here again a stored DNA preparation of a cloning intermediate based on PAC CF1 which was used for the construction of CGT21, a synthetic half *CFTR* locus that was shown to be functional and expressed the intended M470 variant and tag sequences (Laner et al., 2005). Using the silent *Xma*I restriction variant introduced into exon 10 downstream of M470, the EGFP/exon 24 portion of CGT21 was replaced by the missing exon 10 and flanking intronic sequences by ligating a PCR fragment of this region from PAC CF3, resulting in PAC CF1-Met. PAC ligation with the exon 10 fragment resulted in three clones (CF1-Met10-43/-44/-54) containing the novel exon 10 cloning junction as assessed by PCR prior to agarose plug preparation (performed by Sulith Christan).

Amplification by PCR using primer pairs In9F/P77-B (spanning exon 10), In9F/MetR (spanning from intron 9 to part of exon 10), In9F/CF10R, and CF10F/CF10R (both amplify the entire cloned exon 10 sequence, as well as wild-type upstream and downstream sequences, see primers in Table 2) showed that sizes of the PCR products were as expected for the three clones. Sequencing of the cloned exon 10 using primers In9F/P77-B confirmed the right orientation, presence of the corrected V470M polymorphism and that the sequence in all three clones had no PCR derived mutation. Restriction analysis of the In9F/P77-B PCR product revealed the expected bands corresponding to the synthetic restriction sites *Xma*I and *Not*I. Digestion of 1/10 of an agarose plug from all three CF1-Met clones with restriction enzymes *Not*I and/or *Bss*HII also showed the expected fragment sizes of 9.1 kb,

52.1-kb, 91 kb (*Bss*HII) and 8 kb, 15 kb, 52.1 kb, 76 kb (*Bss*HII and *Not*I), respectively. In addition, the expected band sizes were shown for all clones by a semi-quantitative, low cycle number, long-range PCR (LR-PCR) using reaction numbers 2-6 (see Table 1, Figure II.1B, Figure II.2B) covering the entire CFTR sequence cloned in PAC CF1-Met (see map of CGT21 in Figure II.1A), including the 5.1 kb sized reduction of intron 9 in reaction 6 (Figure II.2B). These data indicate that the clones contained the complete insert without rearrangement. As the 31.8 kb band of subclone CF1-Met10-43 (lane 3 of reaction 5 in Figure II.2B, compare Table 1) was very faint compared to the products of the other bacterial subclones, possibly indicating a change in some of the bacteria used for plug preparation, the corresponding clone was not further used.

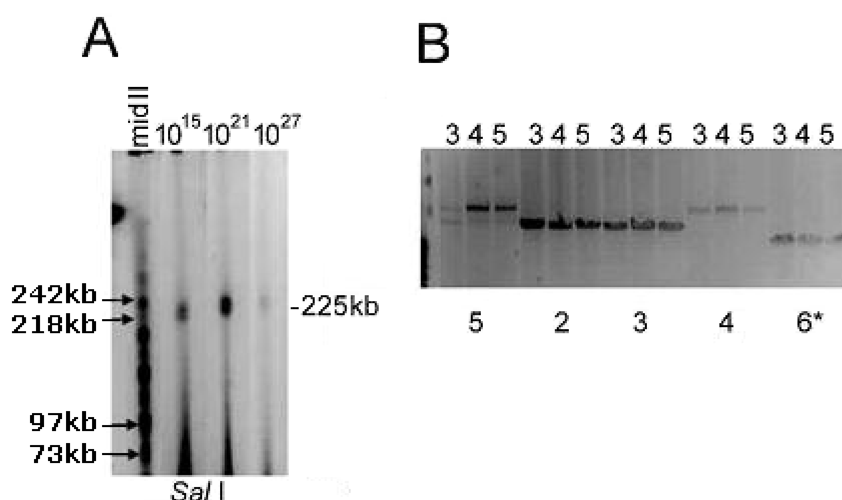

Figure II.2 Stability analysis of the *CFTR* locus cloned in PACs using size mapping of restriction fragments and LR-PCR reactions on pulsed field gels (ethidium bromide stained). **(A)** The PFGE gel shows the *Sal*I inserts of CF225 subclones 5 (left lane), 3 (middle lane), and 2 (right lane) after prolonged culture simulating an expansion to a theoretical yield of 10^{15}, 10^{21}, and 10^{27} *E. coli* cells, respectively. Albeit loading differences, all preparations after 5 (10^{15}), 9 (10^{21}), and 13 (10^{27}) days of growth, show the expected size of 225 kb. The smear at the bottom of the lanes corresponds to *Sal*I digested bacterial DNA (unpurified plugs), midII, lambda multimers (MidRange II marker, New England BioLabs, Ipswich, MA, USA). **(B)** LR-PCR based fine mapping of the *CFTR* locus contained in single cell derived subclones 43 (3), 44 (4), and 54 (5) of PAC CF1-Met. LR-PCR reaction numbers 2-6 (Figure II.1B) are given at the bottom. All three subclones show an identical product size for reactions 2 (18.8 kb), 3 (18.1 kb), 4 (30.2 kb), and 6* (14.6 kb), the latter also indicating the 5058 bp deletion in intron 9 compared to the 19.6 kb wt size of reaction 6. Subclone 43 (3) showed a weak full size product for reaction 5 (31.8 kb) compared to the other subclones and was not used for the construction of CF225. These reactions exemplify cloning stability and suggest presence of genuine *CFTR* loci in the PACs.

After confirming that the *CFTR* sequence cloned in PAC CF1-Met had the same structure as the original PAC CF1, the next step was to fuse CF1-Met PAC with the insert of PAC CF6, which carries the rest of the *CFTR* genomic sequence with

correct exon and intron junction sequences (Ramalho et al., 2004). The cloning strategy was as follows: 1) partial digestion of CF1-Met PAC (clone 44) with *Not*I, total digestion of CF6 with *Not*I followed by dephosphorylation to suppress circularization; 2) separation by pulsed-field gel electrophoresis (PFGE) of *Not*I restriction products and excision of the bands without UV exposure; 3) electroelution of the DNA fragments from the gel slices and mixture at a ratio ~1/1 (CF1-Met/CF6); 4) T4 DNA ligation; 5) electroporation into *E. coli* DH10B; 6) PCR screening of kan^R colonies with primers CFi10fus/R7 (Table 2) specific for the fusion region between the two PACs. One clone, later designated CF225, was found to be positive for the fusion and for STSs of exons 4 and 12 of both PACs.

TABLE 1. SUMMARY OF THE LR-PCR REACTIONS USED FOR STRUCTURAL ANALYSES OF CLONED *CFTR* LOCI

Reaction number (Figure II.1B, II.2B)	Primers	CF1 Prep CF1", CF1-Met 44+55	CF1 Prep CF1-Met 43	CF6 Prep CF6'	CF225 Prep	Product size (kb)
2	CF-37F/ CF-18R	+	+	n.d.	n.d.	18.8
3	CF-18F/ CF-1R	+	+	n.d.	n.d.	18.1
4	CF-1F/CF3R	+	+	n.d.	n.d.	30.2
5	CF3F/CF7bR	+	(+/-)	n.d.	n.d.	31.8
6	CF7bF/CF10R	+*	+*	n.d.	n.d.	19.6/ *14.6
8	CF11F/CF14cR	n.d.	n.d.	+	n.d.	7.4
9	CF14iF/ CF17bR	n.d.	n.d.	+	n.d.	14.1
10	CF17bF/CF21R	n.d.	n.d.	+	n.d.	41.5
11	CF19cF/ CFaM2R	n.d.	n.d.	+	n.d.	41.4
14	CF19cF/aB5R	n.d.	n.d.	+	n.d.	44.0
15	aMF/P86	n.d.	n.d.	+	n.d.	10.5
16	P77/12R	n.d.	n.d.	+	n.d.	0.7
17	SP6/CF1-5R	n.d.	n.d.	n.d.	+	0.3
18	CF6-4F/Sp6	n.d.	n.d.	n.d.	+	0.6

*product with other than wt size

3.2 ANALYSIS OF THE CLONING STABILITY OF CF225

During initial growth of the obtained CF225 PAC master culture, twelve single *E. coli* cell derived subclones were plated and analyzed by PCR to assess cloning

stability. Nine subclones contained the eight tested PCR-STSs covering the locus at positions -18 kb (CF-18F/R), -1 kb (CF-1F/R), exon 3 (CF3F/R), exon 10 corrected/ fusion (CFi10fus/R7), exon 11 (CF11F/R), exon 17 (CF17bF/R), exon 21 (CF21F/R) and the poly A region (CFaF/R; primer sequences are described in Table 2).To further analyze cloning stability, three single cell derived cultures of PAC CF225 were grown for various time periods simulating a potential final yield of 10^{15}, 10^{21}, and 10^{27} *E. coli* cells, each containing up to ~10 completely replicated copies of the construct (Ioannou et al., 1994). A DNA yield of a 10^{15} cell preparation represents a normal laboratory scale required for functional testing. The 10^{21} scale may represent the upper range required for extensive testing in a multicenter gene therapy trial. Larger cell numbers could perhaps represent the range of a continuous production of a DNA based drug for the clinical setting.

Restriction digestion of plug material from the three single cell derived colonies of CF225 showed an identical insert size of ~225 kb (*Sal*I) (Figure II.2A) and identical *Bss*HII fragments of the predicted sizes of 96.5 kb, 83.4 kb, 52.1 kb, and 9.1 kb (not shown), indicating a very high overall cloning stability of the locus. These data indicate a faithful and stable propagation of the *CFTR* locus in PAC CF225 (225.3 kb insert).

3.3 STRUCTURAL ANALYSIS OF CLONED *CFTR* LOCUS

3.3.1 LR-PCR

To analyse the stability of the cloned CF1-Met insert, genomic regions were analysed in detail using LR-PCR spanning the whole insert. For this analysis, a minimum of 5 overlapping PCR products were generated (Figures II.1B, II.2B). Primer pairs and sizes of these 5 and other products used to confirm the structure and internal order within the large DNA insert from PACs CF1-Met10 and CF6, or to analyze end products, are given in Table 1. The PCR products showed identical fragment sizes for all genomic regions from the three different CF1-Met10 clones (Figure II.2B and Table 1). Thus, the long PCR and pulsed field restriction analysis demonstrates presence of the expected structure and a lack of internal rearrangements in the three clones. The data obtained for PACs CF1-Met10 (Figure II.2B, Table 1), CF6 (Table1) and CF225 (Figure II.2A) indicate that the entire *CFTR* gene can be stably cloned in *E. coli*. Occasionally, deletions have been observed under pro-recombinatorial conditions in *E. coli* expressing *rec*E and *rec*T in the PAC

host DH10B, which is RecA negative for the stable propagation of large inserts (Shizuya et al., 1992; Ioannou et al., 1994), as reported for a repetitive region 50 kb 3' to the beta globin locus (Imam et al., 2000). Overall, these structural analyses indicate a very high cloning stability of the *CFTR* locus in *E. coli*, suggesting that a genomic DNA of clinical use can be produced in sufficient quantity and quality.

3.3.2 END SEQUENCING OF LOCUS CF225

To determine the ends of CF225, LR-PCR reactions were carried out with primer pairs SP6/CF1-5R (5' end) and CF6-4F/SP6 (3' end) (Tables 1, 2). These were found to amplify 605 bp and 265 bp products (Figure II.3B), respectively. SP6 hybridizes to a sequence present on both sides of the ligated PAC vector backbone of CF225. The amplified fragments were sequenced and blasted against the hg built 37.1 at NCBI. The reconstructed *CFTR* locus runs from nucleotide position -60651 relative to the start of translation to nucleotide position +9767 relative to the end of translation. Both ends coincide with a *Sau3*AI site, in agreement with the partially digested genomic DNA cloned in the *Bam*HI site of the PAC vector pCYPAC2 in library RPCIP704 (Ioannou et al., 1994).

As a result of the cloning procedure, CF225 has two deletions within introns 9 and 10 representing regions which were not covered by the genuine PACs CF1 (intron 9) and CF6 (intron 10) and were omitted by reconstructing exon 10 and its flanking intron sequences. The resulting 5.1 kb deletion in intron 9 was already known from the functional analysis of CGT21 (Laner et al., 2005) not to affect expression and correct splicing in this region. To precisely locate and determine the extent of those deletions, PCR reactions were carried out with primer pairs In9F/C16D (intron 9/exon 10), and CFi10fus/CF11R (intron10/intron 11) (Table 2), which generate DNA fragments of 735 bp and 773 bp, respectively. The PCR products were sequenced and blasted against published human BAC sequences. In9F hybridizes to nucleotide positions 73794 – 73816, and C16D to nucleotide positions 79552 – 79529 on the *CFTR* genomic sequence (relative to the start of translation). The deletion in intron 9 is located from nucleotide position 74148 to 79204 and is 5058 bp long. CFi10fus hybridizes to nucleotide positions 80085 – 80105, and CF11R to nucleotide positions 107929 – 107952. The deletion in intron 10 runs from nucleotide positions 80281 to 107408 and is 27128 bp long. DNA from HT1080 cells served as a control for the absence of amplification from wt-*CFTR* loci (not shown).

3.4 CO-TRANSFECTION EXPERIMENTS

In order to analyze the functional incorporation of the obtained *CFTR* locus in a *de novo* formed human artificial chromosome (HAC), we employed simple co-transfection experiments with linearized DNA components. HAC formation by co-transfection is not efficient, but has successfully been used to incorporate a *HPRT* gene into a *de novo* formed HAC (Grimes et al., 2001). In addition, it is advantageous not to pre-fabricate a fixed composition regarding the size of the locus, the orientation with respect to the centromere, and the type of centromere included. Indeed, co-transfection of the HAC components separately may to some extent increase the flexibility of the assembly of a HAC, which may adopt some rules of how stable structures need to be composed. On the other hand, the ongoing repair and recombination processes required to generate a stable genetic entity may by chance alter the input DNA. Thus, co-transfections represent a workable tool to initialize HAC formation studies.

Four rounds of co-lipofection of the 225 kb insert of PAC CF225, with the characterized preparation of construct TTE1 (133 kb fragment) containing a duplicated BS selectable marker gene and the EGFP marker, resulted in 185 (white and green) cell clones, 122 of which were expanded and screened by PCR with primers CFi10fus/R7 (Table 2), specific for CF225. Five individual cell clones, BW24, BG32, CG13, DG27, and DG5 were positive for the exon 10 junction region, indicating that only 1 in ~25 cell clones was co-transfected with both CF225 and TTE1 DNA. Although the ratio of intact molecules per liposome preparation was not further assessed in the four individual transfer experiments resulting in the 122 analyzed clones, there is no obvious explanation for this low co-transfection efficiency. Other co-transfections >100 kb regularly approached efficiencies of 1 in ~3-10 clones when equimolar DNA preparations were used under similar conditions, regardless if one or both components carried the BS marker. Successful co-transfections leading to HAC formation were possible at higher efficiency even if one component lacked telomeric repeats, as was the case here for the CF225 insert. Nevertheless, the co-transfections of CF225 still represented a workable means of selecting out stable clones allowing an initial functional assessment of the locus not carrying a selectable marker.

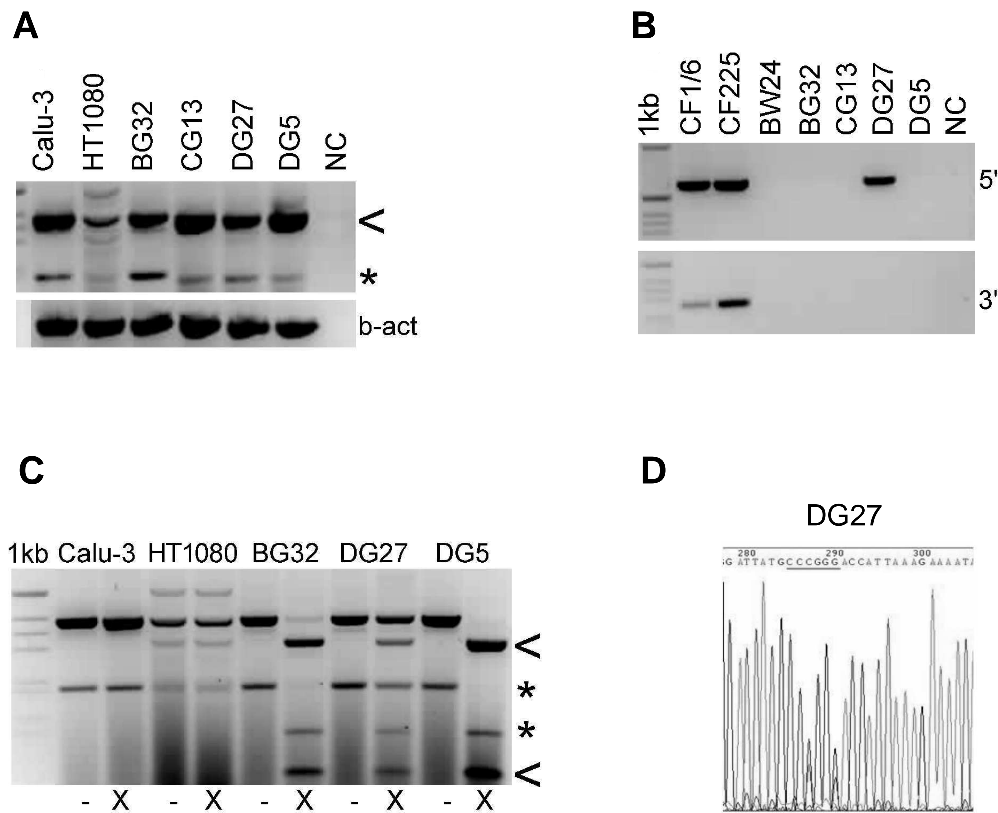

Figure II.3 Expression of CFTR constructs and analysis of locus ends. **(A)** Lines co-transfected with CF225 (225 kb) and TTE1 (133 kb) were analysed with primers B3F/C16D resulting in a 391 bp product composed of endogenous and transgene transcripts (arrowhead). A (normal) fraction of transcripts showing exon 9 skipping is indicated (asterisk). **(B)** End specific PCR reactions SP6/CF1-5R (0.6 kb, 5' end) and CF6-4R (0.3 kb, 3' end) specific for construct CF225 showed that only line DG27 retained the 5' end, whereas all lines obtained from the co-transfections showing presence of the exon 10 region specific to CF225 (5 out of 122 lines) showed loss of the 3' end. Nevertheless, 4 out of 5 lines showed expression of the CF225 specific RT-PCR product as shown in C. **(C)** *Xma*I restriction analysis of RT-PCR products of CF225 lines and comparison with Calu-3 and the weakly expressing HT1080 cells. The products of both controls are not cut with *Xma*I. Varying proportions of the 391 bp products of lines BG32, DG27, and DG5 are cut into 310 and 81 bp bands (arrowheads), indicating presence of functional CF225 transgenes. The minor product of 208 bp indicating (normal) exon 9 skipping is also cut with *Xma*I, which is present in exon 10, leading to a 121 bp band (asterisks). Ethidium bromide stained agarose gels with the 1 kb ladder (Invitrogen, Carlsbad, CA, USA) size standard. **(D)** A section of the electropherogram of the sequencing of the RT-PCR product of line DG27 showing presence of the introduced *Xma*I site CCCGGG sequence (underlined), in addition to the endogenous transcripts of HT1080 showing the wt sequence CCTGGC, both encoding P and G at aa positions 499 and 500.

RT-PCRs were carried out with primers B3F and C16D (Table 2) generating a spliced product of 391 bp between exons 8 and 10, which represents a mixture of products from endogenous *CFTR* genes of the HT1080 cell line and the transgene loci. RNA/cDNA preparations were controlled using β-actin primers (Figure II.3A). All lines showed varying levels of *CFTR* expression after 30 days off selection. To

distinguish between endogenous and transgene expression, the RT-PCR products were digested with *Xma*I cutting the engineered exon 10 from CF225 into two fragments of 310 bp and 81 bp (Figure II.3C). In four cell lines, varying proportions of the CFTR transcript resulted from the transgene, which demonstrated expression levels of the transgene above wt background in most cases, and showed correct splicing (Figure II.3C). Cell line BW24 did not express the transgene (not shown). The expressing cell lines and parental HT1080 cells were further analyzed by sequencing of the RT-PCR products with the same primers and primer CFc3F (Table 2), demonstrating that all lines contained both the 470M polymorphism and the synthetic *Xma*I variant in exon 10, confirming transgene origin. Figure II.3D shows a section of the electropherogram from sequencing of line DG27, evidencing both the *Xma*I site (CCCGGG) expressed from CF225 and the wt sequence (CCTGGC) from the HT1080 loci, both encoding wt amino acids 499P and 500G (corresponding to silent exchanges).

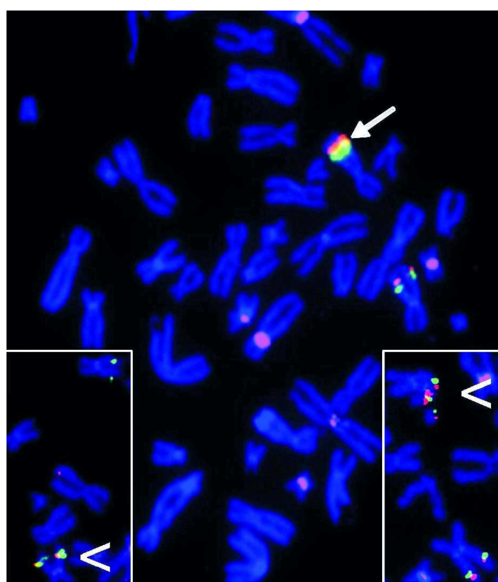

Figure II.4 FISH based HAC detection. Triple colour FISH analysis of line DG27 containing the CF225 (225 kb) and TTE1 (133 kb) cotransfected material integrated into chromosome 7 close to the endogenous *CFTR* locus (arrow). Probe CF1 (red), probe CF6 (green), and traces of probe E1 (pink) staining the centromere 5 alpha satellite DNA and related centromeres of chromosomes 1 and 19 are present at the site of integration. A rare case (1 in 130 metaphases) of fragility at the site of integration suggests presence of multiple copies (right inset). Vector probe *rsf* spanning the BS marker (red) is present at the integrated locus marked by the CF6 probe (green) and the cen5 probe E1 (pink) (left inset).(Data generated by Sulith Christan, reproduced here with permission).

To check the integrity of construct CF225 in the clonal cell lines, PCR reactions were carried out with primer pairs SP6/CF1-5R for the left vector/CFTR junction of 605 bp, and CF-4F/SP6 for the right CFTR/vector junction of 265 bp (Table 2). Of the 5 cell lines which were positive for the exon 10 junction PCR, line DG27 has kept the left end of CF225, confirmed by sequencing the PCR product. All 5 lines were negative for the right end junction PCR (Figure II.3B) and for two other LR-PCR reactions extending approximately 2 kb and 3 kb from the right end into the *CFTR* locus (not shown), suggesting that 3' DNA of CF225 was lost in all four expressing lines. Loss of locus ends may have occurred during integration or subsequent propagation.

Triple colour FISH analyses of these cell lines after 30 days of growth on and 30 days off BS selection revealed either integration of the CF225 locus into host chromosomes, or integration and truncation in all five clonal cell lines. No HACs carrying the *CFTR* locus were observed. Clonal line DG27 showed a stable co-integration close to the endogenous *CFTR* gene on chr7 (arrow in Figure II.4), which was positive for CF1, CF6, and *rsf* signals on and off selection. Weak signals for E1 were regularly visible at the site of integration. Line BG32 revealed a distal/telomeric integration into a chromosome (non-7), which was positive for CF1, CF6, and *rsf*, but not for E1 signals, indicating that the alpha satellite DNA was either not co-transfected or lost. Cell line DG5 showed integration of CF1, CF6, and E1 signals in a distal position of chr19q, and line CG13 showed integration of CF1 and CF6 portions in the p arm of a metacentric chromosome (non-7) accompanied by truncation. The truncated portion containing the endogenous centromere and signals for CF1 and CF6 was stably maintained whereas the p arm portion positive for signals E1 and CF6, but not CF1, was frequently lost. In cell line BW24 only CF6 sequences were detected on a small truncated chromosome. Overall, we conclude that CF225 and the E1 centromere did not efficiently form a stably replicating structure together. Instead, rare stable clones were selected out which contained at least the BS marker and various portions of locus CF225 lacking the very 3' end, which nevertheless showed expression of the tagged exon 10 sequence in 4 out of 5 lines obtained.

CHAPTER III

OVERALL DISCUSSION

CHAPTER III. OVERALL DISCUSSION

CF gene therapy, with delivery of the correct *CFTR* gene to cells, promises a great benefit for all CF patients, irrespective of the type of mutations. However, classical gene therapy to CF has had limited success due to immune response or short term transgene expression of cDNA delivered by viral vectors or cationic lipids due to silencing. These limitations could be overcome by delivering the complete *CFTR* gene with relevant regulatory regions within its genomic context on non-integrating human artificial chromosomes. Thus, for gene therapy success, a stable and functional *CFTR* locus containing all exons, introns, and regulatory elements should be inserted into a human artificial chromosome (HAC) vector that can be delivered to cells.

CGT21, a genomic construct containing about one half of the *CFTR* locus from which expression and correct splicing was demonstrated (Laner et al., 2005), is a functional gene with 10 exons encoding a non-functioning CFTR protein derivative. Due to its reduced size and the tagged exon, such construct can ease certain transfer studies.

Here, we aimed at producing and analysing the stable incorporation of a reconstructed *CFTR* locus (including all exons and introns, and most regulators) on a *de novo* HAC, which could be used for gene therapy. The *de novo* approach based on the transfection of "naked" DNA molecules to the cells of the patient offers a technically feasible strategy towards long term gene therapy, provided sufficient numbers of large constructs can be produced in a functional form and delivered.

We describe the successful generation of a large construct containing the *CFTR* locus with a reduced size (PAC "CF225"), constructed from characterized CFTR PAC resources, with a functionally optimized polymorphism and a silent restriction variant in exon 10. Other experiences were carried out in the same laboratory using the 296.8 kb sized BAC 5A (Figure II.1A) covering the entire wt *CFTR* locus with all regulators [work performed by Lucia Rocchi (Rocchi et al., 2010)].

For reconstructing the tagged *CFTR* locus, CF225, we adopted a technically simple approach based on a "rare cutter" (*Not*I) restriction site present at the PAC vector boundary, thus allowing "conventional" cloning on a genomic scale, followed by electroporation of the entire *CFTR* fusion PAC into *E. coli* DH10B cells. To our

knowledge, cloning of PAC fragments of this size, which is based on intact DNA preparations, represents a novel method to assemble genomic loci.

To define the *CFTR* locus, we used our previous data, describing that the human *CFTR* gene and its adjacent genes localize individually and differentially to distinct nuclear regions, according to their transcriptional activity. This suggests that the principal *CFTR* regulatory information is contained within the limits of the two adjacent genes, i.e., < 283 kb (Zink et al., 2004; Sadoni et al., 2008). In addition to developmentally regulated alternative 5' exons (Mouchel et al., 2003) numerous DNase I hypersensitive sites (DHS) have been described and analysed within this region (McCarthy and Harris, 2005; Ott et al., 2009a). Most of these are included in CF225, namely DHSs that lie at: -20.9 kb (to the start of translation), in introns 1, 2, 3, 11, 16, 17a, 18, 20 and 21 and 3' to the gene at +5.4, +6.8, +7, and +7.4 kb relative to the end of translation. DHSs not present in CF225 likely belong to the adjacent genes, or are in the reduced region of intron 10, including the DHS that has been shown to be active in intestinal cells (Ott et al., 2009a), and DHSs at +15.6 kb, +17.2 kb, and +20.1 kb (to the end of translation). The latter are placed in the vicinity of the start position of a ~17 kb transcript mapped in opposite direction of the *CFTR* gene (source: hg build 37.1 at NCBI), running in from the intergenic region between *CFTR* and *CORTBP2* towards the end of the *CFTR* transcript.

Similar transcripts in the opposite strand can be found in the 3' region of many genes, for example the genes *FOXP2*, *MDFIC*, *CAV2*, *CAV1*, *MET*, *CAPZA2*, just to name a few in the vicinity of the *CFTR* locus (unpublished observation). The common presence of such 3' transcripts suggests an important genetic function, for example for gene regulation, formation of a chromatin domain, or for stability.

By co-transfecting CF225 and TTE1, we obtained five BS resistant cell lines which, however, did not show a *de novo* formation of HACs. All obtained integrated cell lines have lost the 3' end of CF225 DNA, which could include loss of DHS at +5.4, +6.8, +7 and +7.4 kb. Although these DHS are potentially absent and the ones at +15.6 kb and downstream are not in the construct, it was possible to detect the *Xma*I specific, correctly spliced transcript in RT-PCR products from 4 out of 5 cell lines, albeit at varying levels relatively to the endogenous *CFTR* genes of HT1080.

Due to the poor co-transfection efficiency observed with PAC CF225, it remains unknown whether the CF225 locus is complete and replication-competent (autonomously) when ligated to a centromere. It seems possible that additional 3' sequences, such as an origin of replication, or chromatin barriers separating gene expression from centromere function could be required on a pre-fabricated HAC. PAC CF225 contains three *Not*I sites at both locus ends and in intron 10, which is 27

kb reduced in size compared to the wt locus. Thus, the possibly critically short 3' end, or the lacking DHS in intron 10 can easily be altered by cloning suspected regulatory regions into the *Not*I sites, representing an interesting system to verify expression regulation and stability on a HAC in suitable cell or animal models.

To study incorporation of three distinct versions of the *E. coli* cloned CFTR locus into *de novo* HACs, co-lipofection experiments were carried out in HT1080 cells. A workable number of 24 and 25 clones, respectively, revealed a *de novo* formation of a stable HAC when using either a short, BS selected construct CGT21 (151 kb, linearized with I-*Sce*I; Figure II.1A) and the BS selected cen 17 construct B2T8 (~200 kb, linearized with I-*Sce*I) (Laner et al., 2005), or when using the long, not selected wt locus 5A (~300 kb *Sal*I insert; Figure II.1A) and the BS selected cen 5 construct TTE1 (133 kb, linearized with *Not*I) (Rocchi et al., 2010). In contrast, the more elaborate analysis of 122 clones from the co-transfection of the midsized, non selected locus CF225 (225 kb, *Sal*I insert; Figure II.1A) with the BS selected cen 5 construct TTE1 (133 kb, linearized with *Not*I) did not reveal HAC formation. Only 5 integrated lines positive for CF225 were obtained. Although the numbers are not sufficient to conclude that CF225 is less HAC competent than 5A, it is tempting to speculate that CF225 is prone to instability. While the larger 5A construct has kept both locus ends in 2 individual cell lines (out of 25 analysed lines, which represent <25 individual lines, due to double picked ones, which were excluded when identified) including an integrated and the HAC line (Rocchi et al., 2010), all 5 integrated lines of the midsized locus CF225 (out of 122 lines) have lost the 3' end of the locus. Nevertheless, the specific RT-PCR analysis demonstrated that CF225 is expressed in 4 out of 5 cell lines, and that the introduced *Xma*I variant in exon 10 can be used to distinguish between transgene and endogenous transcripts. It is possible that CF225 underwent a 3' trimming process to generate a stable structure, which may have reduced the co-transfection efficiency and thus HAC incorporation.

The HAC present in cell line EC14 [derived from the co-transfection of locus 5A and the BS selected cen 5 construct TTE1 into HT1080 cells (Rocchi et al., 2010)] was highly stable on and off BS selection and contained the entire wt-*CFTR* locus. Moreover, the cen 5 alpha-satellite DNA present on the HAC bound CENP-A, indicating formation of an active centromere (Rocchi et al., 2010). Accordingly, the HAC in line EC14 represents the first *de novo* formed human artificial chromosome carrying the entire *CFTR* locus. Cell line EC14 showed expression of the *CFTR* locus above the endogenous background on and off selection, suggesting that the functional locus was transferred. Unambiguous proof of expression from the *CFTR* genomic construct that localizes in the vicinity of the active centromere is not

possible at present, since high copy numbers are likely to be present on the HAC, resulting in a larger distance to the centromere of some copies. Moreover, HAC material integrated into chr1 could also contribute to expression (Rocchi et al., 2010).

It was previously demonstrated that PAC cloned, >100 kb sized human alpha-satellite arrays with ~99% identical 2 kb repeats did not result in internal recombination or deletion during a prolonged growth period of 2 x ~400 *E. coli* generations in a colony re-plating experiment (Schindelhauer and Schwarz, 2002). Here, we provide extensive structural data including a set of LR-PCR reactions covering the *CFTR* loci from different cloning sources and show stability. Moreover, we could confirm cloning stability after the prolonged growth periods in fluid culture, which would be required to test and produce a HAC based DNA therapeutic product. Overall, the cumulative data presented here suggest that the construction of a pre-fabricated CFTR-HAC based on the defined resource clones is feasible. These results are thus promising for the development of a HAC based therapy.

CHAPTER IV

MATERIALS AND METHODS

CHAPTER IV. MATERIALS AND METHODS

1. PAC CLONES COVERING THE *CFTR* LOCUS

Details of the isolation of clones carrying the *CFTR* locus from PAC library RPCIP704 (Ioannou et al., 1994), the sequencing procedure, and primers are available online (Ramalho et al., 2004). The PAC vector used both in preparation of PAC library RPCIP704 and in this work was pCYPAC2 (Figure IV.1).The ends of PAC CF1 were mapped by pulsed field gel electrophoresis (PFGE) and amplified using primers In9F and T7 (3') or directly sequenced with PAC end primer SP6 (5'). The ends of PAC CF6 were mapped using primers P77 and CF12R (5') and primers aMF and P86 in a long PCR reaction (3'). For construction of the intermediate construct CF1-Met, primers MetF and MetR (Table 2) were used for the V470M exchange by site directed mutagenesis in a plasmid containing an intron 9 and exon 10 portion during construction of CGT21, and primers E10XcF and CF10NMR (Table 2) for the introduction of the silent *Xma*I variant and intronic *Not*I site for PAC ligation. Introduced primers and PCR products were sequenced in the resulting clones. Construct CGT21 (EMBL/Genbank accession number BN000167), and the centromere construct TTE1 containing a 116 kb sized alpha-satellite array of chromosome 5 in vector pTT (26-kb) were described elsewhere (Laner et al., 2005). PCR products were sequenced and blasted to the human genome sequence (build 37.1). The expected size of the insert was confirmed by pulsed field gel analysis.

2. *E. coli* GROWTH AND AGAROSE PLUG PREPARATION

E. coli DH10B strain (F *mcr*A Δ(*mrr-hsdR*MS-*mcr*BC) (Φ80d*lac*ZΔ*M15*) Δ*lac*X*74* *deo*R *rec*A1*ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Sm^R) *end*A1 λ *nup*G) was grown in LB broth or agar medium. PAC clones were selected with 30 µg/ml kanamycin. Telomerized PAC clone TTE1 was selected with both 30 µg/ml kanamycin and 50 µg/ml ampicillin.

For large scale growth simulation, single cell subclones 2, 3 and 5 of the master culture of CF225 were established at day 1, grown in 50 ml LB cultures at 37℃ and tested for STS content with follow ups during subsequent growth phases, indicating full stability. For the prolonged growth periods, the subclones were grown in 1 l of rich, buffered LB medium at 30℃, from which agaros e plugs containing ~10^{15} cells

were prepared at day 5. Subsequently, 1 ml of subclones 2 and 3 was transferred to 1 l of fresh medium every other day, resulting in a theoretic number of ~10^{21} and ~10^{27} bacteria at day 9 and 13, respectively, when agarose plugs were prepared.

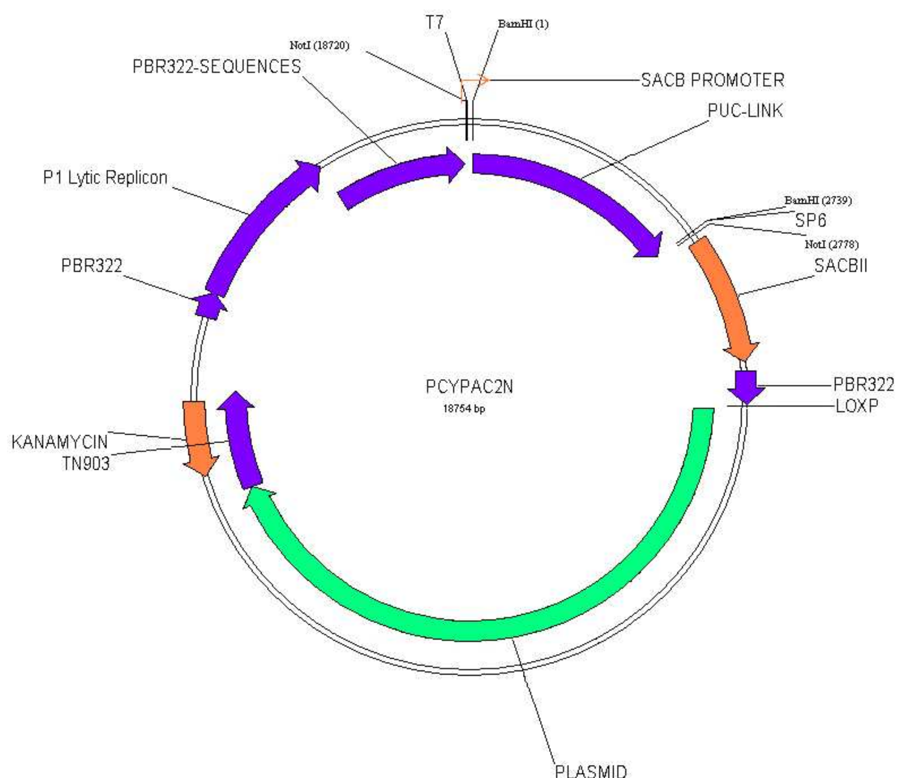

Figure IV.1 A schematic of PAC vector pCYPAC2. The pCYPAC2 vector has been constructed for the cloning of large DNA fragments following electroporation. This vector has been constructed by removing the stuffer fragment from the pAd10SacBII vector (Pierce et al., 1992) and inserting a pUC plasmid into the *Bam*HI cloning site. The pUC-Link is a stuffer fragment, being replaced by insert DNA during cloning. (http://bacpac.chori.org/pCYPAC2.htm).

3. PREPARATION OF INTACT DNA

Long DNA preparations in agarose plugs were carried out according to the protocol of Smith, Klco and Cantor (Smith et al., 1988), supplemented by purification steps to remove *E. coli* fragments and damaged DNA fractions from the circular DNA preparations as described (Schindelhauer and Cooke, 1997).

TABLE 2. OLIGODEOXYNUCLEOTIDES USED IN THIS STUDY

Primer	Sequence (5'-3')	Location	Reference	Used for
P77-B	GGTCGAGCTTGACATTGTAGG	pCYPAC2 (reverse)	This work	PCR, End Sequencing
P86	TGGGCGATCTGCCGTTTCGATCC	pCYPAC2	This work	End mapping
T7	TAATACGACTCACTATAGGG	pCYPAC2	Schindelhauer, 2002; Laner, 2005	PCR
R7	CCTCTCCCTATAGTGAGTCG	pCYPAC2 (reverse complement of T7)	This work	PCR
SP6	ATTTAGGTGACACTATAG	pCYPAC2	Anand, 1991	LR-PCR End sequencing
CF1-5R	GTTCCAATTCTATAAGATTATCAG	CFTR (5' region of CF1)	This work	LR-PCR End mapping/ sequencing
CF-37F	CGTGTTAGGCTGATTTTGCAGC	CFTR (5' region)	This work	LR-PCR
CF-37R	CGACCAGCACATAACAACTCAGC	CFTR (5' region)	This work	LR-PCR
CF-18F	GATGTCCTGCAACTGGCAGAG	CFTR (5' region)	This work	LR-PCR
CF-18R	CAGAGATCTACATGTGAGGGC	CFTR (5' region)	This work	LR-PCR
CF-1F	CTCAGAGAGTTGAAGATGGCG	CFTR (5' region)	This work	LR-PCR
CF-1R	GTGAGTGAACTCCAAGGGTGG	CFTR (5' region)	This work	LR-PCR
A1R	CGAGAGACCATGCAGAGGTC	CFTR (exon 1)	This work; Laner, 2005	RT-PCR
CF3F	CTTGGGTTAATCTCCTTGGA	CFTR (intron 2)	This work; Zielenski,1991	LR-PCR
CF3R	ATTCACCAGATTTCGTAGTC	CFTR (intron 3)	This work; Zielenski,1991	LR-PCR
CFc3F	GGGATAGAGAGCTGGCTTC	CFTR (exon 3)	This work	RT-PCR Sequencing
CF4F	TCACATATGGTATGACCCTC	CFTR (intron 3)	This work; Zielenski,1991	PCR
CF4R	TTGTACCAGCTCACTACCTA	CFTR (intron 4)	This work; Zielenski,1991	PCR
CF7bF	AGACCATGCTCAGATCTTCCAT	CFTR (intron 6)	This work; Zielenski,1991	LR-PCR
CF7bR	GCAAAGTTCATTAGAACTGATC	CFTR (intron 7)	This work; Zielenski,1991	LR-PCR
B3F	AATGTAACAGCCTTCTGGGAG	CFTR (exon 8)	Ramalho,2002	RT-PCR Sequencing
In9F	CAAGTAGCAGGTGAAGCAAGTGC	CFTR (intron 9)	This work	PCR Sequencing
CF10F	GCAGAGTACCTGAAACAGGA	CFTR (intron 9/ exon 10)	This work; Zielenski, 1991	PCR
CF10R	CATTCACAGTAGCTTACCCA	CFTR (intron 10)	This work; Zielenski, 1991	PCR LR-PCR

TABLE 2. OLIGODEOXYNUCLEOTIDES USED IN THIS STUDY (CONT.)

Primer	Sequence (5'-3')	Location	Reference	Used for
MetF*	GACTTCACTTCTAATGATGATT ATGGGAGAACTGG	CFTR (intron 10)	This work	PCR, V470M exchange
MetR*	CCAGTTCTCCCATAATCATCAT TAGAAGTGAAGTC	CFTR (exon 10)	This work	PCR, V470M exchange
E10XcF*	GGATTATGCCCGGGACCATTA AAGAAAATATCATCTTTGG	synthetic/ CFTR (exon 10)	This work	PCR, *Xma*I site
CF10NMR*	CGGTGGTACGCGTGCGGCCG CCCTAACATTTACAGCAATAA	synthetic/ CFTR (exon 10)	This work	PCR, *Not*I/*Mlu*I site
C16D	GTTGGCATGCTTTGATGACGC TTC	CFTR (exon 10, reverse)	Ramalho, 2002	PCR Sequencing RT-PCR
CFi10fus	GTGACTGCAATTCTTTGATGC	CFTR (intron 10)	This work	PCR Sequencing
CF11F	CAACTGTGGTTAAAGCAATAGT GT	CFTR (intron 10)	This work; Zielenski, 1991	PCR
CF11R	GCACAGATTCTGAGTAACCATA AT	CFTR (intron 11)	This work; Zielenski,1991	PCR Sequencing
CF12F	GTGAATCGATGTGGTGACCA	CFTR (intron 11)	This work; Zielenski, 1991	PCR
CF12R	CTGGTTTAGCATGAGGCGGT	CFTR (intron 12)	This work; Zielenski,1991	PCR
CF17bF	TTCAAAGAATGGCACCAGTGT	CFTR (intron 17a)	This work; Zielenski, 1991	PCR
CF17bR	ATAACCTATAGAATGCAGCA	CFTR (intron 17b)	This work; Zielenski, 1991	PCR
CF21F	AATGTTCACAAGGGACTCCA	CFTR (intron 20)	This work; Zielenski, 1991	PCR
CF21R	CAAAAGTACCTGTTGCTCCA	CFTR (intron 21)	This work; Zielenski, 1991	PCR
CFaF	CTAGGGTGATATTAACCAGGG	CFTR (poly(A)-region)	This work	PCR
CFaR	GAGGCTTGAAGACATTATGCT AG	CFTR (poly(A)-region)	This work	PCR
CF6-4F	CTCTGTGAAGGAGGTTCTAAG AAC	CFTR (stop, + 9.5 kb)	This work	LR-PCR End sequencing
AcF	GCACTCTTCCAGCCTTCC	β-actin	This work; Laner, 2005	RT-PCR
AcR	AGAAAGGGTGTAACGCAACTA AG	β-actin	This work	RT-PCR

* Modified primers

4. CONSTRUCTION OF CF225

4.1 IN GEL RESTRICTION DIGESTION AND DEPHOSPHORYLATION

For partial digestion of CF1-Met PAC clones, 1 ml of *Not*I buffer with 150 U of enzyme (New England BioLabs, Ipswich, MA, USA) was added to 7 agarose plugs (~20 µg) while for total digestion of CF6, 600 U of enzyme in 500 µl reaction buffer with 1x BSA was added directly onto a slice containing ~20 plug equivalents (~100-µg in 2 ml). The plugs were left overnight at 0ºC. The following day, another 200 U of *Not*I was added directly on the CF6 slice. Digestions were carried out for 1 h at 37ºC for the CF1-Met plugs and for 4 h at 37ºC in a wet chamber for the CF6 plugs. The partial digestion of the CF1-Met PACs was stopped on ice by adding 10% 0.5 M EDTA, pH 7.9, and later NDS buffer (0.5 M EDTA, pH9; 1% *N*-Lauroylsarcosine) containing proteinase K (10 µg/ml), incubated at 55ºC overnight, while CF6 was immediately dephosphorylated as follows. After removal of *Not*I buffer, the CF6 plug slice was washed with bidistilled water, placed on ice, and incubated with 460 µl of 5x calf intestinal phosphatase (CIP) buffer containing 20 µl CIP (New England BioLabs, Ipswich, MA, USA), which was directly added onto the plug slice and incubated for 2h. Incubation for 30 min at 37ºC was stopped on ice using 250 µl of 0.5 M EDTA, pH 7.9. CIP was inactivated overnight with NDS buffer containing proteinase K (10 µg/ ml) at 55ºC in a humid chamber. After equilibrating in 0.5x TAE, the slice was loaded on a 1% agarose pulsed-field gel.

4.2 PFGE SEPARATION AND DNA ISOLATION

After a 20 h run at 6 V/cm with a switch time of 1- 30 s in 0.5x TAE at 12ºC (CHEF DRII, Bio-Rad, Hercules, CA, USA), the PAC DNA bands were cut from the gel without UV exposure, and kept at 0ºC. DNA was recovered from gel slices by electroelution using a Bio Trap BT1000 (Biometrics, Schleicher & Schuell, Dassel, Germany) placed in a CHEF DRII or III PFGE chamber in 0.5x TAE.

4.3 LIGATION AND ELECTROPORATION

The ligation reaction was carried out with 200 µl of eluate, 1x T4 DNA ligase buffer, and 20 µl of T4 DNA ligase (New England BioLabs, Ipswich, MA, USA), at

12ºC, overnight. Reaction mixtures without ligase were used as controls. 4 µl of the ligation reaction was added to 5 µl of electrocompetent *E. coli* DH10B cells (ElectroMAX™ DH10B™ Cells, Invitrogen, Carlsbad, CA, USA) in a volume of 80 µl of bidistilled water and electroporated (Bio-Rad Gene Pulser II) at 1.2-1.4 kV, 100 Ω, and 25 µF using 0.1 cm gap cuvettes (Bio-Rad, Hercules, CA, USA) chilled on ice. 500 µl of warm S.O.C. medium was immediately added to the cuvette and the content transferred to sterile 10 ml white cap tubes containing 5 ml of LB medium for growth for 1 h at 37ºC with moderate shaking. Spun bacteria were spread on LB agar containing 30 µg/ml kanamycin (Sigma, Munich, Germany), and incubated for a minimum of 24 h at 37ºC.

4.4 *E. COLI* SCREENING

Individual colonies were picked into 30 µl of TTE buffer (0.01% Triton X-100, 20-mM Tris-HCl, pH8, 2 mM EDTA, pH8), heated 1 min at 95ºC and spun for 15 min at 10000 rpm. 2 µl of supernatant was used in a final volume of 50 µl for a PCR of 30 cycles with an annealing temperature of 55ºC with primer pair CFi10fus/R7 (Table 2). 2-µl of ligation reaction was used for the positive control. Aliquots of the PCR product were run on a 1% standard + 1% low melting agarose gel.

5. VERY LONG PCR

Agarose plug sections with purified, intact PAC template of ~10 µl were added to a total reaction volume of 50 µl containing tuning buffer (1x Eppendorf), 0.5-mM dNTPs, 0.5 µl $Mg(OAC)_2$, 0.2 mM primers, 2 U Taq polymerase, and 2 U of the TripleMaster Enzyme Mix (Eppendorf). The programme has an initial denaturation at 92 ℃ for 30 sec, 6-9 cycles of denaturation for 12 sec, annealing at 60-64 ℃ for 1-min, and elongation at 66℃ for 15-45 min (20-60 kb), and a final extension at 66℃ for 5 min. 10-20 µl of the gelly product (0.2% LMP agarose) is mixed with 10 µl loading buffer (Ficoll) and analyzed on a pulsed field gel.

6. GENERATION OF STABLE HT1080 LINES

Linearized DNA was isolated using appropriate restriction enzymes (New England Biolabs). *Not*I was used for TTE1, and *Sal*I for the CF225 insert. PFGE separation, excision without UV illumination, and electroelution was as described (Laner et al., 2005) with the modification of placing the BioTrap elution chamber in a CHEF DRII apparatus.

6.1 CELL CULTURE

HT1080 cells were grown in DMEM (PAA Laboratories, Pasching, Austria) supplemented with 10% FCS (PAA Laboratories), 1% Glutamine 100X (Gibco, Karlsruhe, Germany), and 2% (v/v) penicillin/streptomycin (Gibco, Karlsruhe, Germany) at 37ºC and 5% CO_2.

6.2 LIPOFECTION

10-cm tissue culture plates containing ~50% confluent HT1080 cells were washed with PBS (PAA Laboratories, Pasching, Austria). For each plate, 6 µl Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) and 294 µl Opti-MEM I (Gibco, Karlsruhe, Germany) were mixed and incubated for 5 min at RT. Variable volumes of DNA eluate were gently mixed with Opti-MEM, resulting in a volume of 300-µl, which was gently added to the Lipofectamin/Opti-MEM mixture. The tube was turned twice and incubated for 20 min at RT. The solution was directly added onto PBS rinsed cells using wide bore plastic Pasteur pipettes to avoid DNA shearing. Plates were incubated at 37ºC for 12 h, the cells washed with medium, and incubated for 1 day without selection. Medium supplemented with 4 µg/ml blasticidin S (InvivoGen, Toulouse, France) was added at day 3 and changed every other day.

6.3 CLONE EXPANSION AND ISOLATION

Transfected plates were screened by bright field and fluorescence microscopy using an Axiovert 10 (Zeiss, Oberkochen, Germany) in a dark room equipped with a 100 W Hg lamp and filters for blue excitation and green detection of EGFP. Individual

BS-resistant cell clones were isolated using cloning rings, and expanded in 12-well dishes, 25 and 75 cm^2 flasks for PCR screening, growth on and off selection, DNA extraction, RNA extraction, freezing, and FISH analyses. DNA for PCR screening was extracted using lysis buffer (0.1 M Tris pH 8.3, 5 mM EDTA, 0.2 M NaCl, 0.2% SDS, 10 µg/ ml proteinase K) and ethanol precipitation.

7. EXPRESSION ANALYSIS AND SEQUENCING

7.1 RT-PCR

Total RNA was isolated from confluent BS-resistant cells grown in T25 flasks using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol, with the additional DNase digestion (Ambion, Austin, TX, USA). The reverse transcription (RT) reaction was also performed according to the Invitrogen protocol, using SuperScript III One-step RT-PCR kit. Primer sequences used for RT-PCR are shown in Table 2. Efficiency of RNA extraction and the RNA levels in the various samples were controlled by RT-PCR with 1 µl of each RNA sample to amplify a β-actin fragment of 385 bp using primers AcF and AcR (Table 2). Primers B3F/C16D were used to amplify a 391 bp product indicating correct splicing, and a minor product of 208 bp indicating skipping of exon 9. B3F/C16D were also used for analyzing a silent *Xma*I variant present in transgene CF225 as follows. To distinguish CF225 and endogenous CFTR transcripts in the respective lines, 15 µl of the RT-PCR reactions were digested in a 25 µl reaction with 20 U of *Xma*I resulting in 310 and 81 bp fragments derived of the transgene (and a reduction of the minor 208 bp band to 121 bp from exon 9 skipping of the transgene). Fragments were analysed on 1% standard + 1% low melting agarose gels and images registered on an UV Gene Genius BioImaging System digital analyser (Syngene, Cambridge, England). RT-PCR products corresponding to the main transcript (without exon 9 skipping) were cut out from agarose gels, purified and sequenced.

8. FISH ANALYSES

Fluorescence *in situ* hybridization (FISH) was carried out as described (Laner et al., 2004) after 30 days on and off BS selection. After initial screening, a minimum of 20 (mostly > 30) metaphases were analyzed for each growth phase. The probes

used were *rsf* for the vector sequence including the BS marker (Laner et al., 2005) labelled by PCR with biotin-16-dUTP (Roche, Mannheim, Germany) and Cy3.5 avidin, nick translated PAC inserts CF1 (biotin-16-dUTP, Cy3.5, red) and CF6 (digoxigenin-11-dUTP, FITC, green), nick translated 2.6 kb *Eco*RI repeats excised of PAC B2 containing the cen 17 alpha-satellite array (digoxigenin-11-dUTP, FITC), and a PCR generated, nicktranslated probe E1 of vector TTE1 (DEAC, pink), hybridizing to cen 5 and alpha-satellite arrays on chromosomes 1 and 19. Signals were visualized on an Axiovert 200 microscope and digitally captured (Zeiss, Oberkochen, Germany).

CHAPTER V

FUTURE DIRECTIONS

CHAPTER V. FUTURE DIRECTIONS

Before we can speak of the future, we must start with the past and then get to the present. Past, present and future are connected. So we shall start by describing our past unsuccessful experiences and then suggest ways to turn them into achievements.

In this work attempts were made to construct a HAC, which was one of the objectives of the study. First, we started by ligating both the linearized *CFTR* reconstructed locus CF225 and the linearized BS selected cen 5 TTE1 construct. The next step was electroporating the whole structure into *E. coli* DH10B as we had done for cloning CF225. All our attempts were fruitless, probably because it is very difficult to electroporated an artificial chromosome of ~360 kb into *E. coli* cells without breaking it. Another possible explanation is that CF225 is not competent to originate an autonomous replicating unit together with the centromere proficient TTE1 (see below).

We also focused on producing a *de novo* HAC inside the HT1080 fibrosarcoma cell line following a different strategy, since our first rounds of co-transfection (CF225 and TTE1) resulted in integration of the CF225 construct into endogenous chromosomes. The CF225 construct carries no selection and it could be a handicap to produce the HAC, so we decided to insert a selectable marker into the construct in order to facilitate the formation of a CF HAC. In the first assays, the strategy was to ligate a PGK-neo cassette, i.e., the neomycin resistance gene under the control of the PGK promoter, which drives gene expression both in bacteria and in mammalian cells, to the CF225 construct, followed by electroporation into *E. coli* cells. Again our attempts met with failure, most likely due to the large size of the structure being electroporated.

We did not give up and another strategy was chosen. This consisted in disrupting the *sac*BII gene in the pCYPAC2 vector (Figure IV.1) with a zeocin™ resistance cassette from the pZeoSV plasmid (Invitrogen), which provides selection in *E. coli* and mammalian cells. The *sac*BII gene was essential for establishing the PAC library RPCIP704 (Ioannou et al., 1994) but afterwards it is no longer needed, so it can be disrupted without causing any damage to the cells carrying the PAC vector. In order to accomplish the task proposed above the following steps were taken: 1) PCR amplification of *sac*BII 5' and 3' homology arms (DNA fragments about 500 bp long) using specific primers; 2) digestion of both homology arms and plasmid

pZeoSV with appropriate restriction enzymes; 3) double ligation of *sac*BII homology arms on both sides of the zeocin resistance cassette; 4) Electroporation of both CF225 and the zeocin resistance cassette flanked by *sac*BII homology arms into *E. coli* modified strain SW106 (Warming et al., 2005), which contains the lambda prophage recombineering system based on Red gene-mediated homologous recombination (Lee et al., 2001), made electrocompetent (and previously grown at 42ºC for 20 minutes for induction of the Red genes). It was expected that the *sac*BII gene in PAC vector pCYPAC2 would be substituted by the zeocin resistance cassette flanked by *sac*BII homology arms after homologous recombination has occurred. Although we did not succeed in ligating the zeocin resistance cassette to the *sac*BII homology arms we still think this is a valuable strategy to bear in mind for the future.

Although no *de novo* HAC was obtained from co-transfecting CF PAC CF225 (225.3 kb; Figure II.1A) and TTE1 into HT1080 cells we know that this HAC strategy is promising since a HAC was obtained from co-transfecting the unselected CF BAC 5A (~300 kb; Figure II.1A) and TTE1 into the same cell line, despite in this case an integration of HAC material into chromosome 1 also occurred (Rocchi et al., 2010). The results observed in this work raised the question whether the CF225 locus is capable of autonomous replication when coupled to a centromere or even stable considering it lost its 5' end in 4 out of 5 cell lines created and the 3' end was lost from all cell clones upon integration into the cell genome. Blackledge and colleagues (Blackledge et al., 2007) proposed the DHS located at +15.6 kb to the end of translation as the 3' boundary of the *CFTR* gene functional unit and so it seems reasonable to extend the 3' end of locus CF225 from +9.8 to +15.6 kb relative to the translation endpoint. To further complete locus CF225, reduced in intron 10 by 27 kb relative to the wt locus, which causes loss of one DHS from CF225, the missing sequence from intron 10 should also be included in the reconstructed *CFTR* locus. Both alterations are feasible as *Not*I sites are present at the 3' end and in intron 10 of CF225 and these could be used to add additional sequences. Then the novel *CFTR* locus could be retested again.

The production of a functional CFTR HAC has major advantages over other types of gene therapy based on delivery of the *CFTR* gene to CF affected cells by viral vectors, such as high-capacity, autonomous replication, maintenance of the transgene in the host cell and long-term regulated expression in a tissue-specific manner (Basu and Willard, 2005; Grimes and Monaco, 2005), all necessary characteristics in the implementation of a successful gene therapy for CF. The work accomplished here raised the expectations in fulfilling every CF patient's dream of

having a cure for CF available to them. The results obtained here and the possibilities that arise from them are very important in the field of CF therapy and give hope that in the future it will be possible to obtain a single cure for all CF patients independently of the *CFTR* mutation they carry.

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