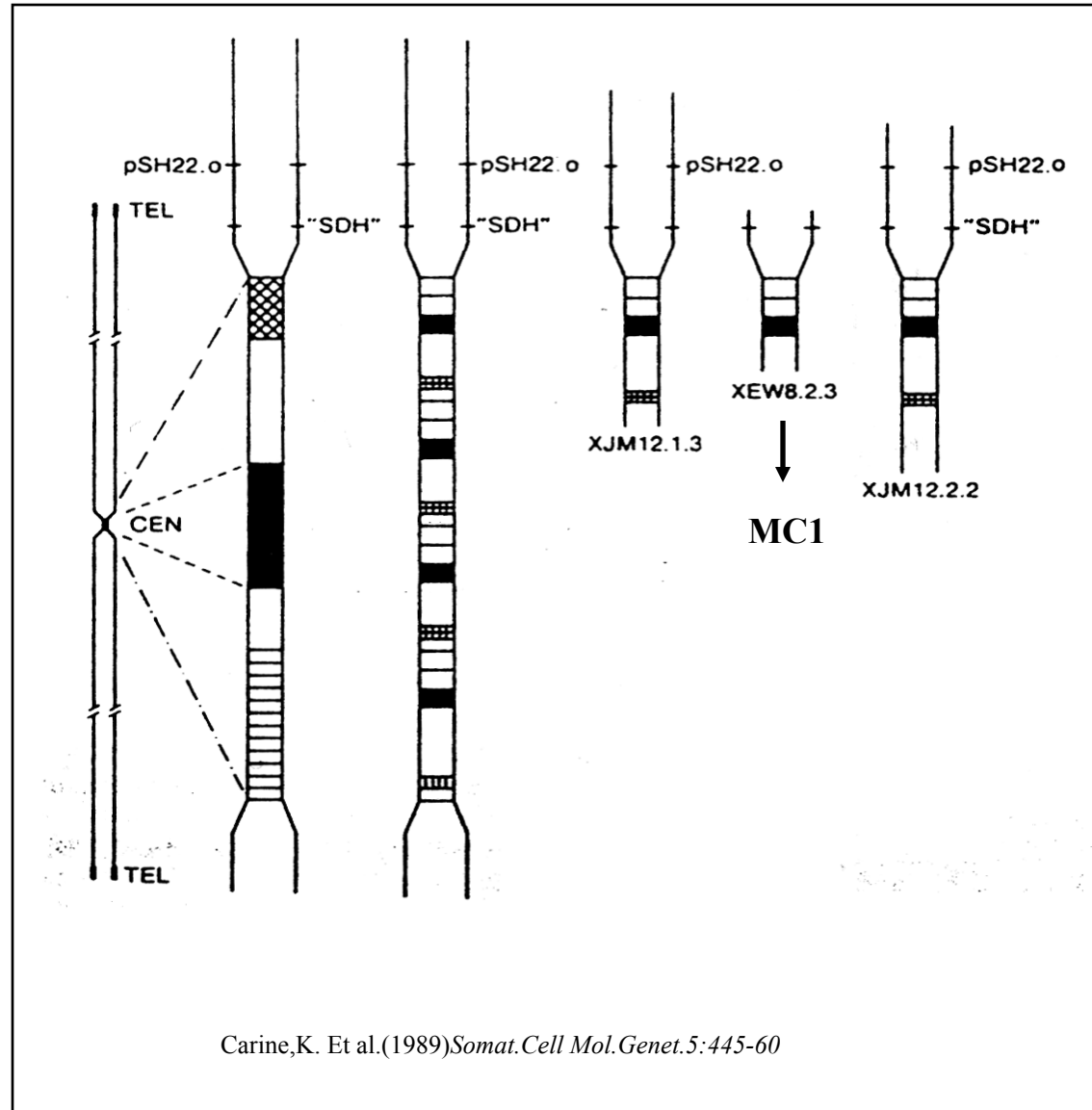


Minicromosomi ottenuti mediante irraggiamento con raggi γ

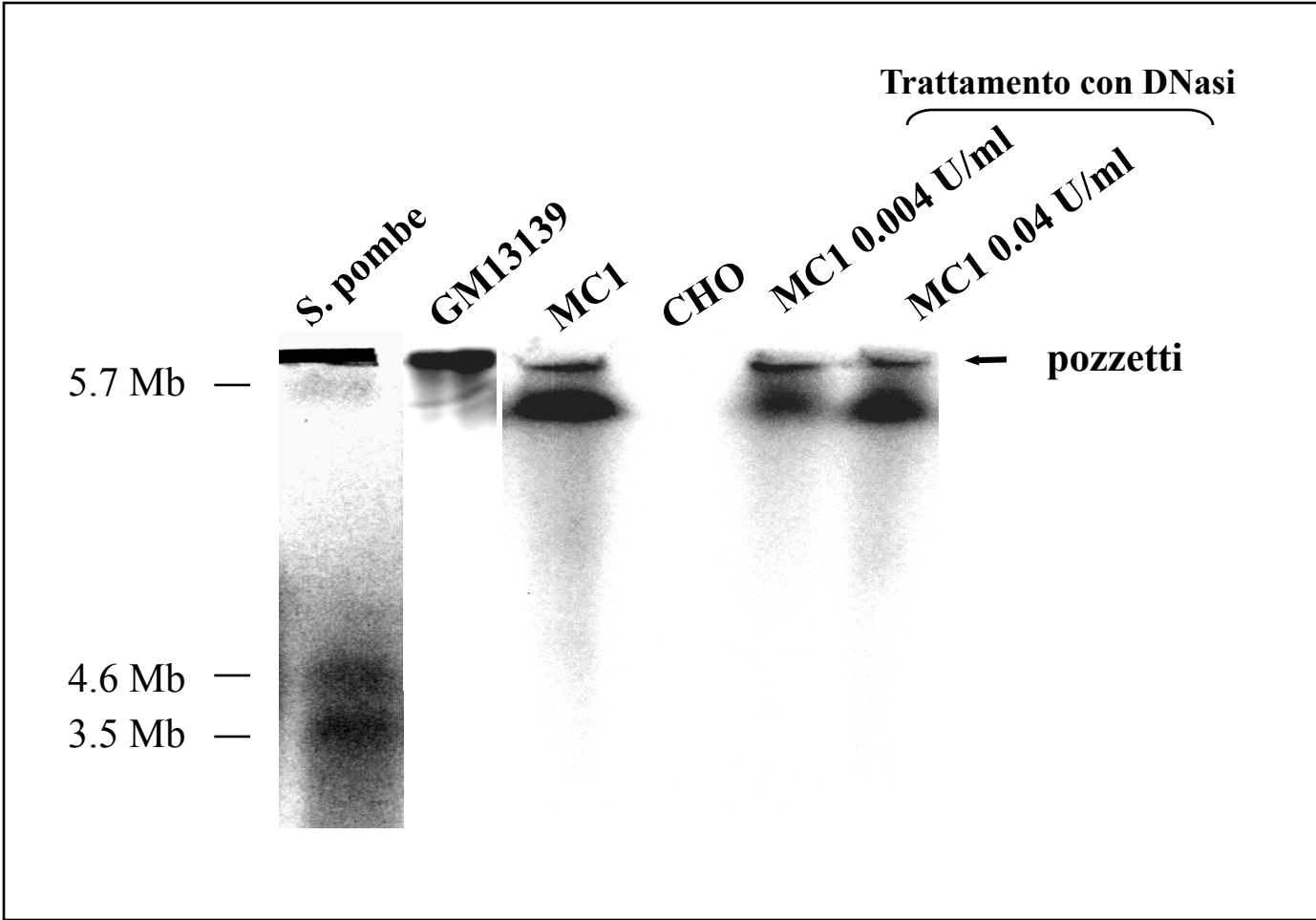


steps toward the assembly of a CFTR-HAC

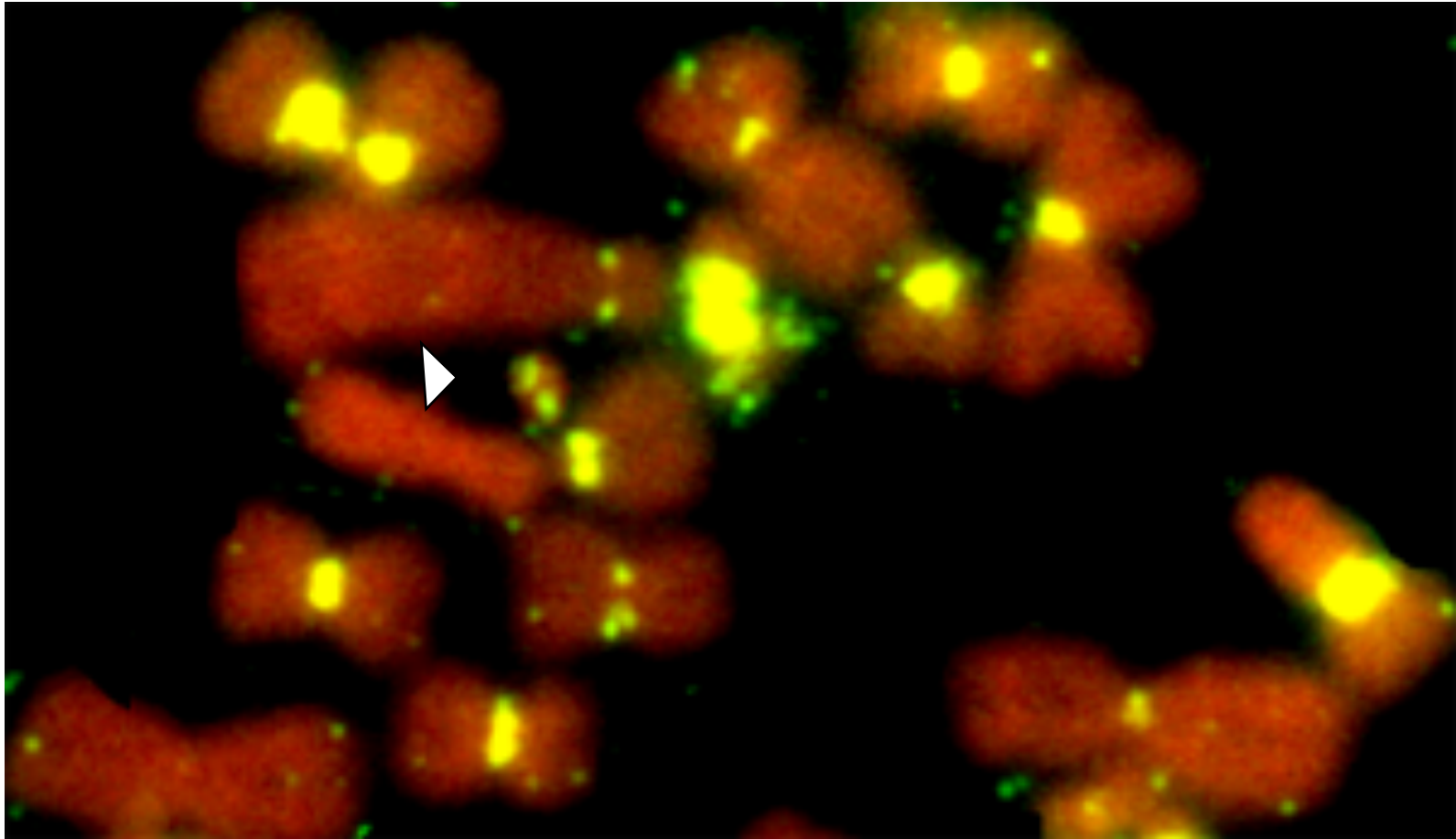
- **structural analysis of the MC1 minichromosome**
 - **determination of the size and the structure (linear/circular)**
 - **analysis of the centromeric and pericentromeric regions**
 - **functional analysis of the centrome**

- **cloning of the CFTR gene and functional analysis**

Separazione di MC1 mediante PFGE



two distinct telomeric signals have been detected by FISH



DNA centromerico

- satellite classico

- satellite 1
- satellite 2
- satellite 3

- satellite alfa

- satellite beta

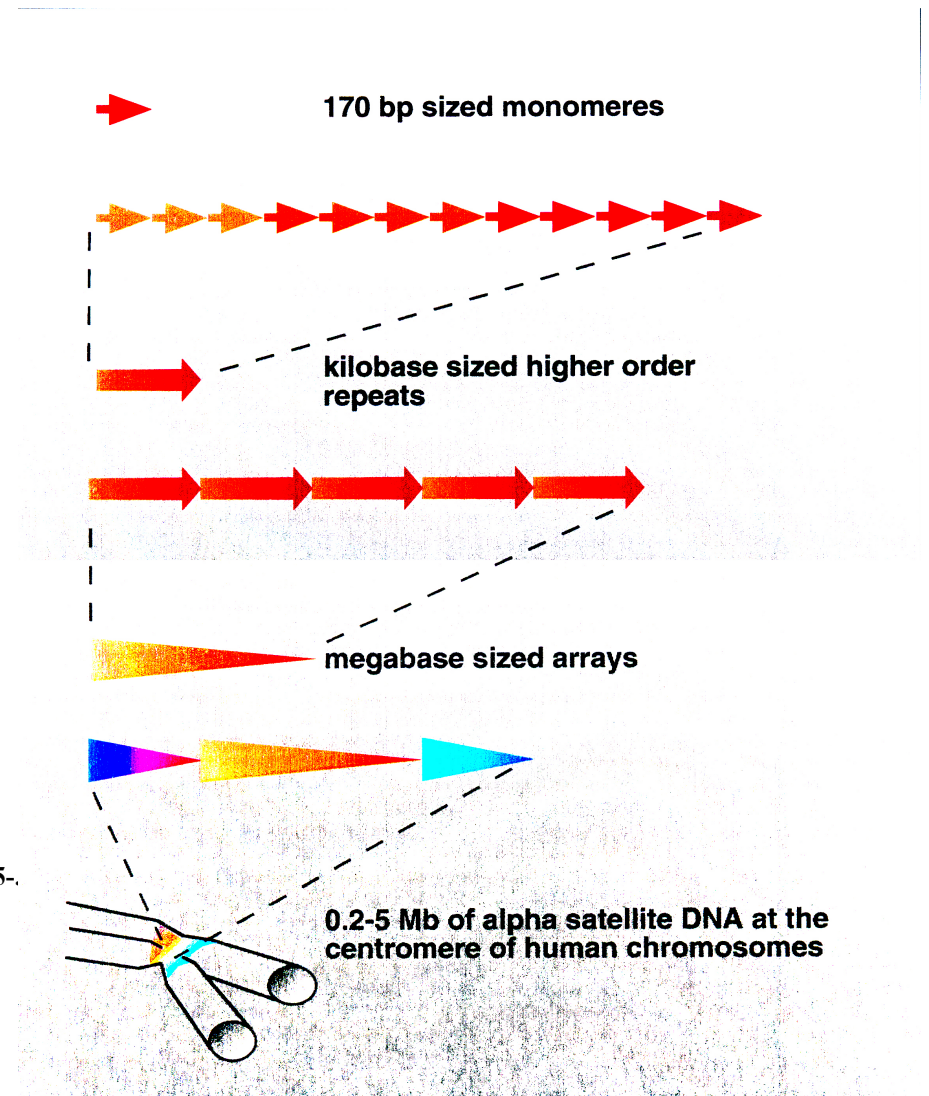
- satellite gamma

- satellite 42-bp

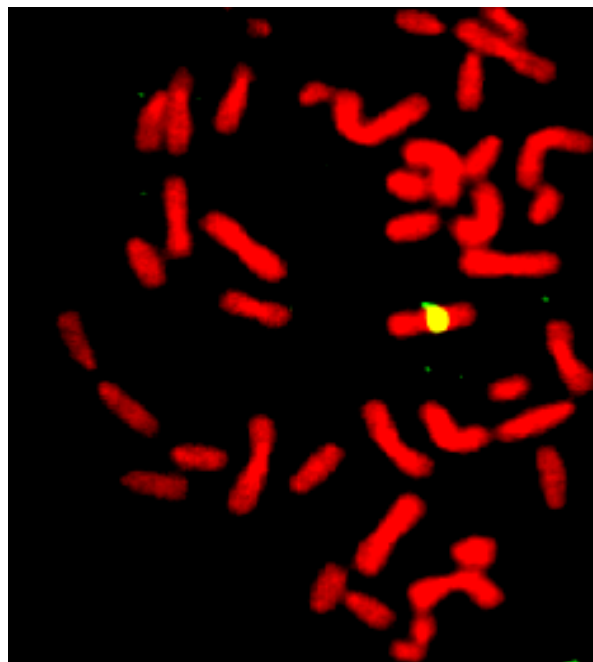
- satellite Sn5

Famiglie sopracromosomali di DNA alfoide

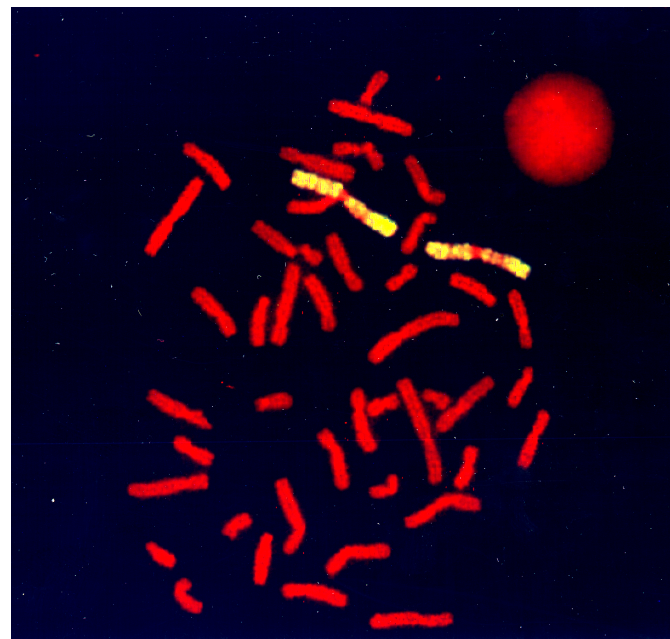
Famiglie sopracromosomali	Cromosomi	Tipo di monomeri	Tipo di organizzazione dei monomeri
1	1, 3, 5, 6, 7, 10, 12, 16, 19	tipo J	... - J1 - J2 - ...
2	2, 4, 8, 9, 13, 14, 15, 18, 20, 21, 22	tipo D	... - D1 - D2 - ...
3	1, 11, 17, X	tipo W	... - W1 - W2 - W3 - W4 - W5 - ...
4	13, 14, 15, 21, 22, Y	tipo M	... - M1 - ...
5	5, 7, 13, 14, 19, 21	tipo R	... - R1 - R2 - ...



MC1 contiene prevalentemente DNA centromerico e pericentromerico del cromosoma 1



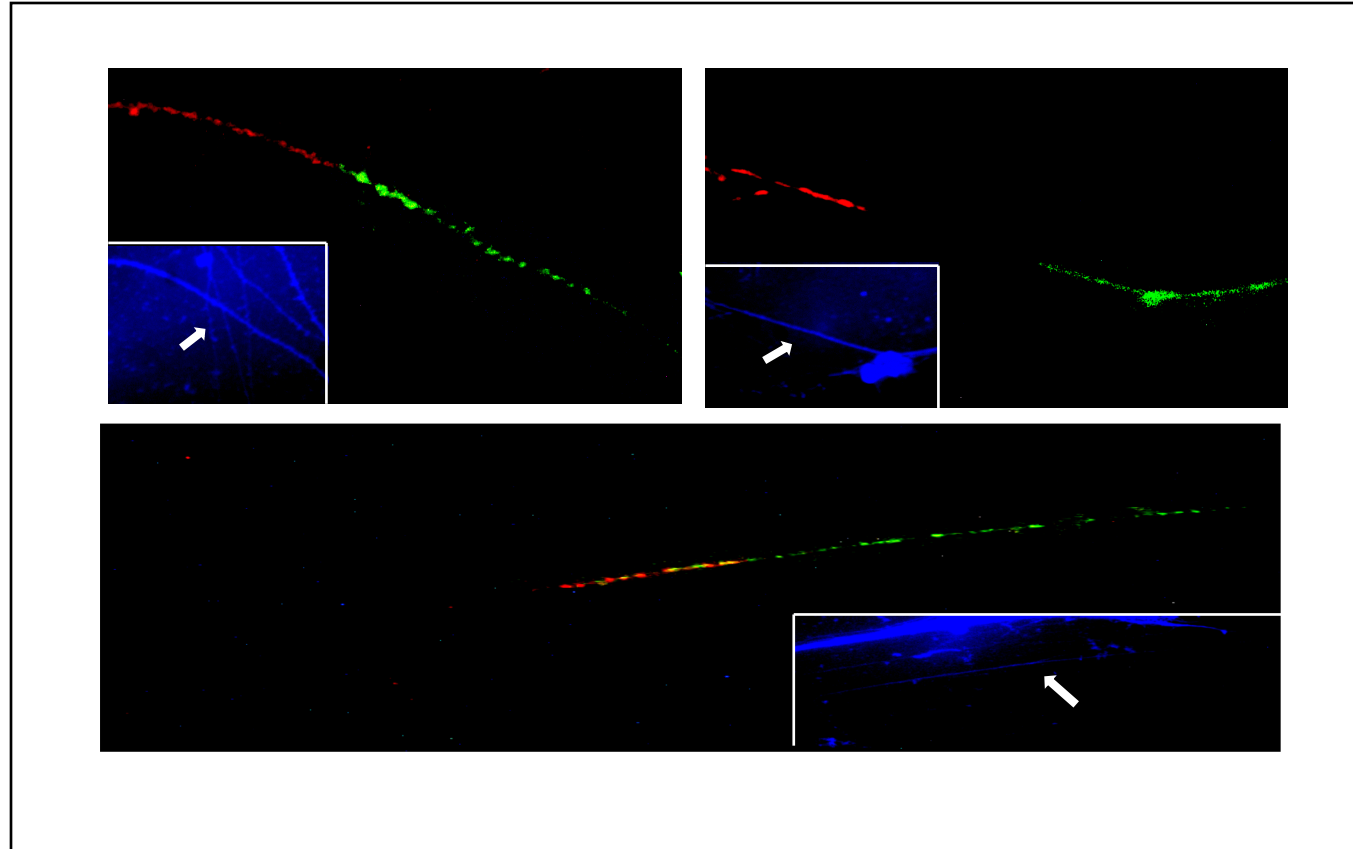
Sonde **MC1-DNA**



Cotosome 1

Analisi della regione centromerica e pericentromerica di MC1 mediante FISH su fibre distese

D1Z5 red
Sat2, green

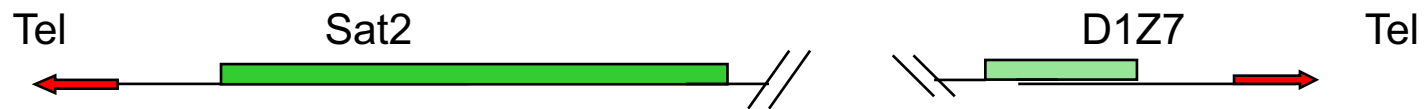
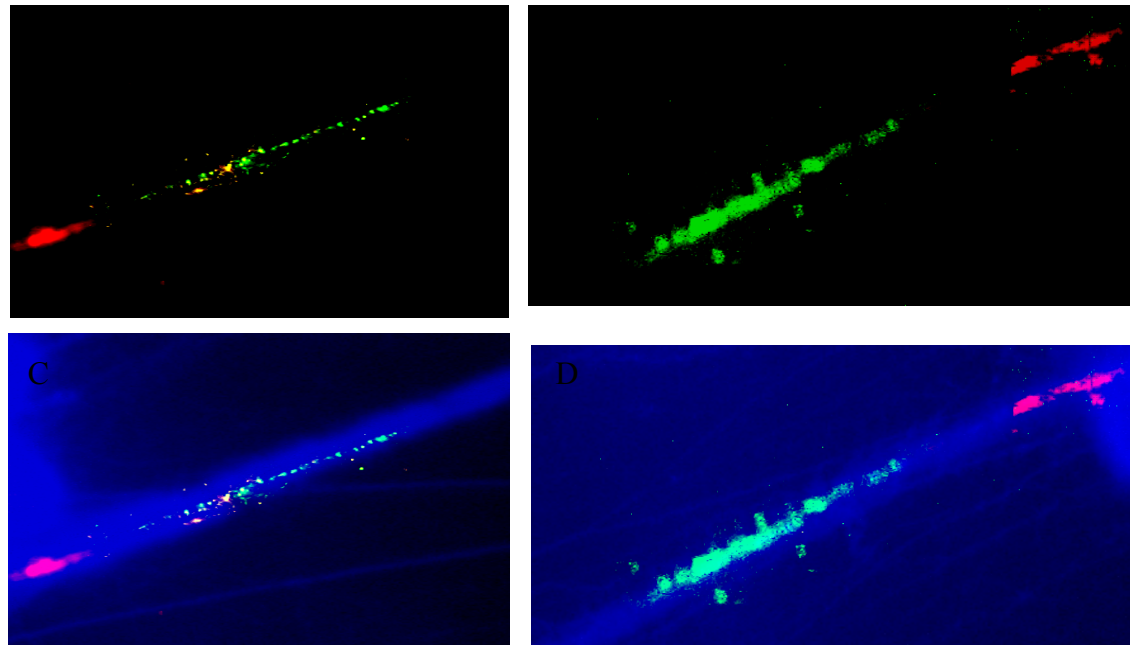


D1Z5 red
Sat2, green

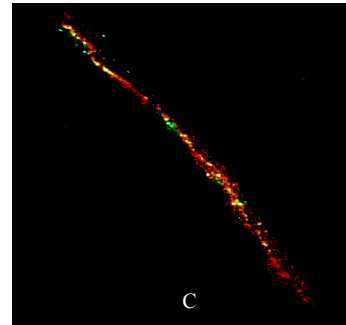
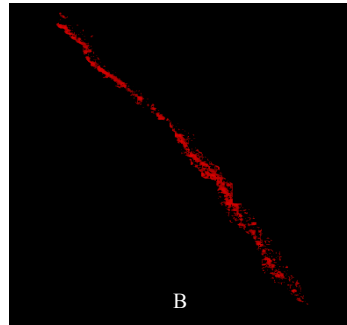
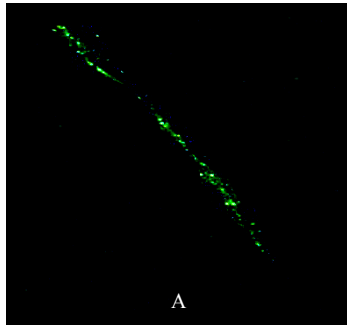
D1Z5, chromosome 1 specific alphoid DNA

D1Z7, alphoid present on chromosomes 1, 5 and 19

Analisi delle regioni terminali di MC1 mediante FISH su fibre distese

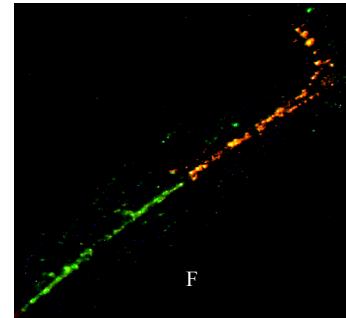
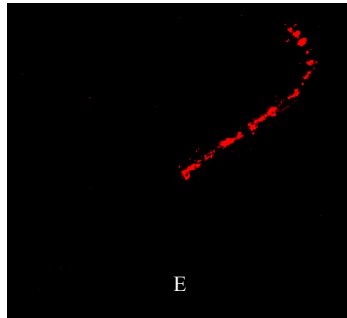
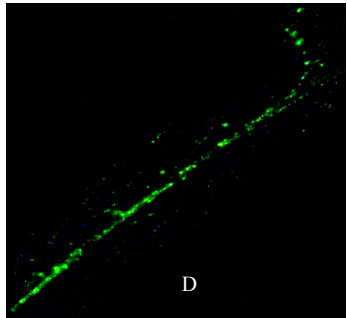


Analisi delle proteine centromeriche di MC1



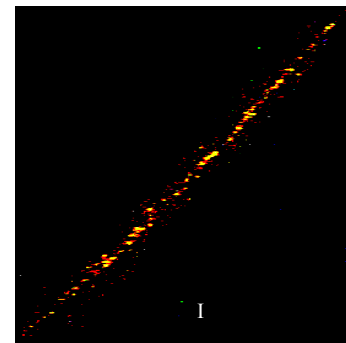
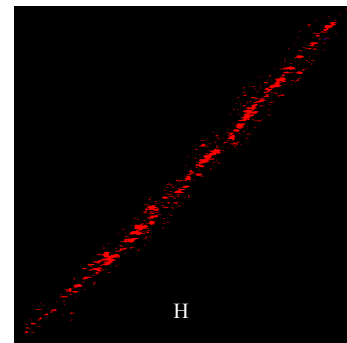
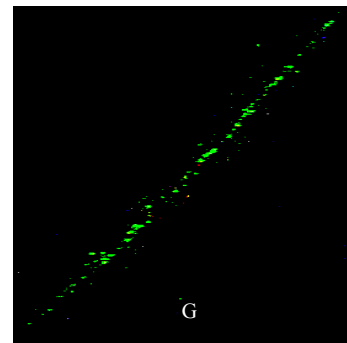
Alfoide/FITC

CREST/Cy3



Sat2/FITC

CREST/Cy3

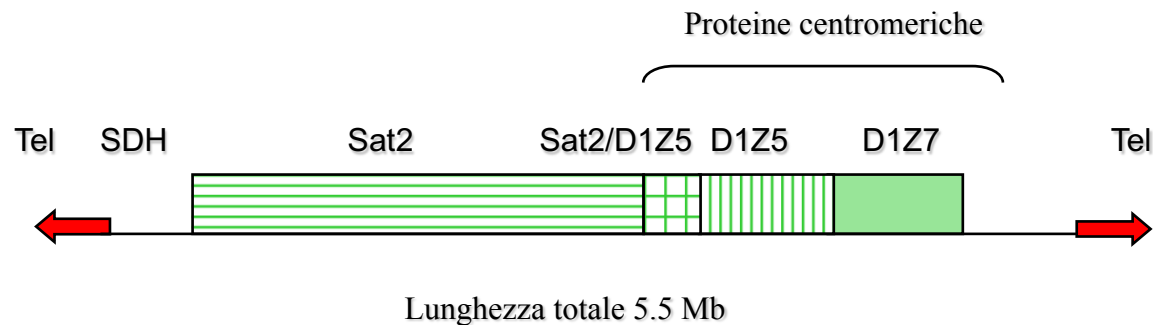


Alfoide/FITC

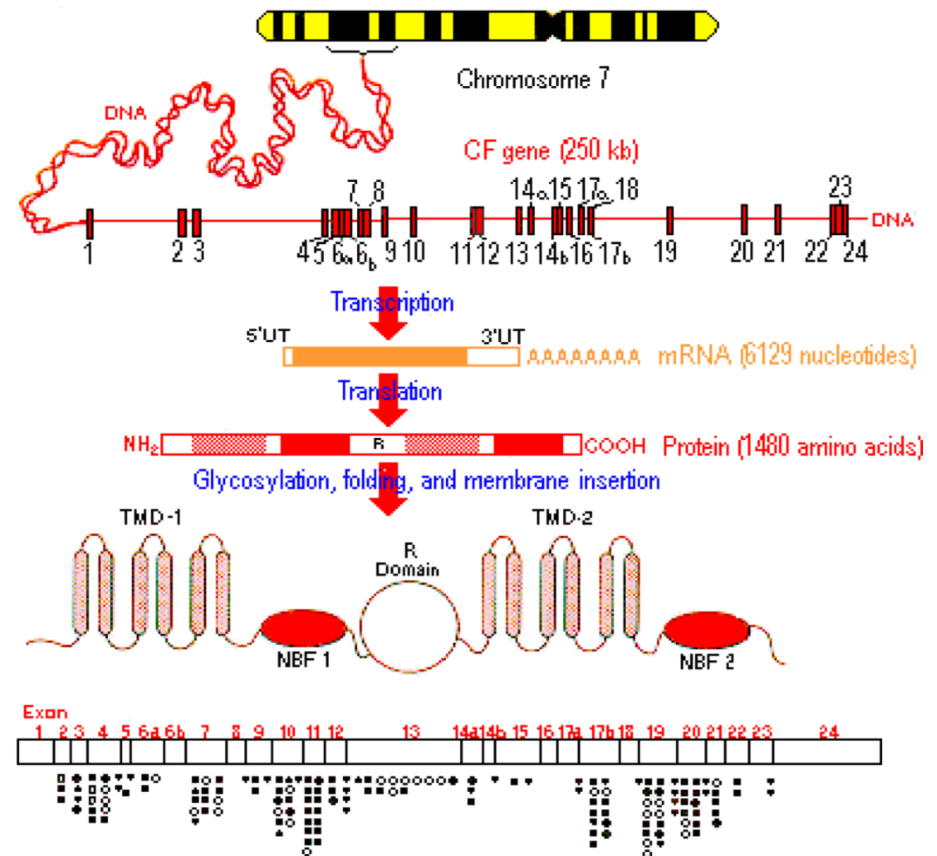
CENP-F/Texas Red

STRUTTURA DI MC1

- MC1 è una molecola lineare di 5.5 Mb
- la molecola è costituita prevalentemente da DNA satellite 2 (3.5 Mb) e da una piccola frazione di alfoide (1.1 Mb)
- il satellite 2 è ridotto del 35% rispetto al cromosoma 1 mentre l' alfoide si è ridotto circa del 65%
- è presente una particolare regione di interspersione Sat2/alfoide
- sono presenti sequenze subtelomeriche ad entrambe le estremità di MC1
- le proteine centromeriche si legano ad MC1

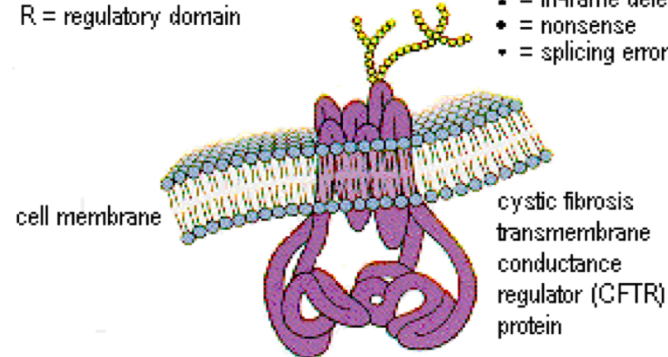


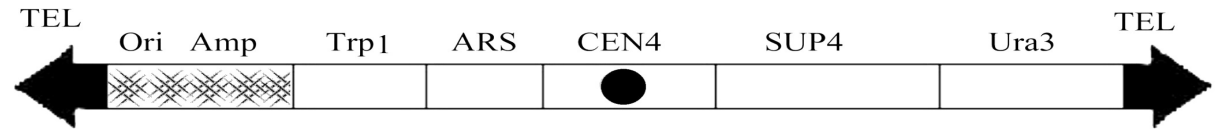
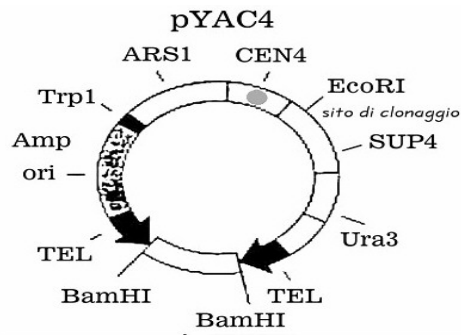
CFTR gene and protein



TMD-1, TMD-2 = transmembrane domains
 NBF-1, NBF-2 = nucleotide-binding folds
 R = regulatory domain

mutation types
 • = in-frame deletion ▪ = missense
 • = nonsense ◦ = frame-shift
 • = splicing error

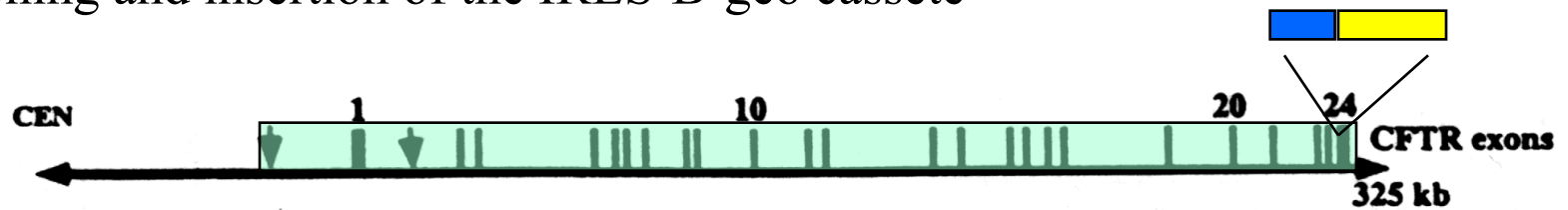




Yeast Artificial Chromosome (YAC)



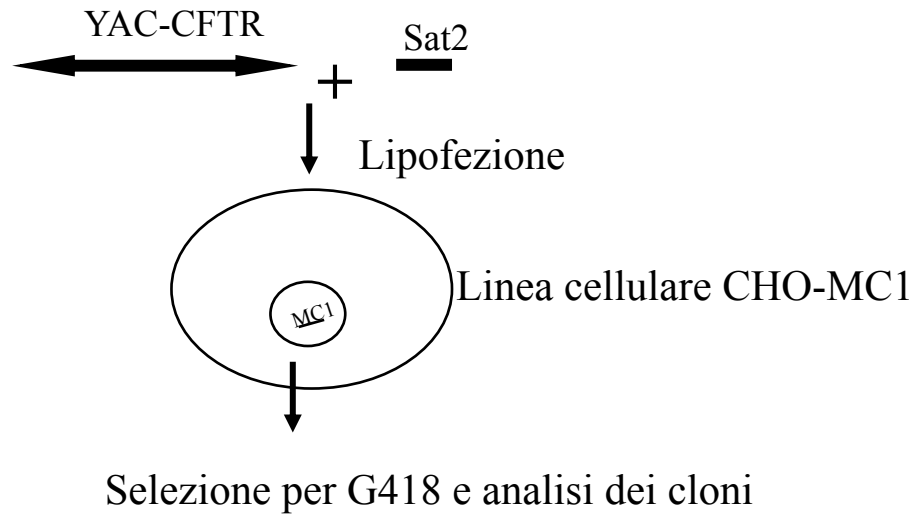
CFTR cloning and insertion of the IRES-B-geo cassette



yCFIRES dicistronic YAC, which contains the human CFTR locus and a picornaviral IRES- β -geo cassette fused to the 3' end of the CFTR gene

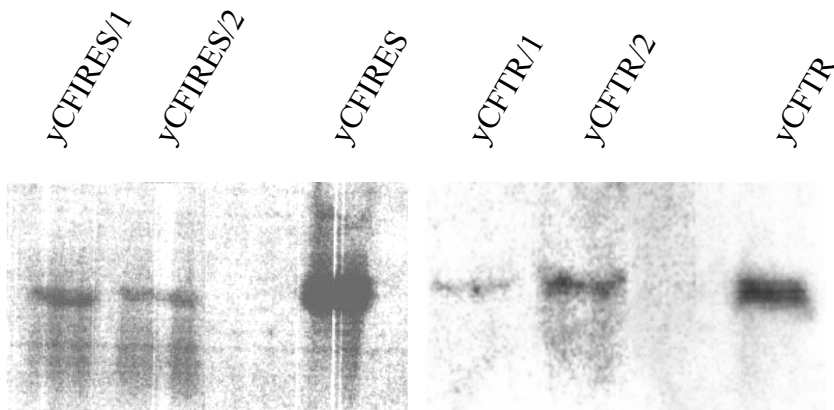
Costruzione di MC1-CFTR: strategia I

Trasfezione degli YAC yCFTR e yCFIRES in CHO-MC1



Cloni MC1-CFTR ottenuti mediante lipofezione

DNA	reagente	cloni neoR	cloni CFTR	MC1-CFTR
yCFTR/Sat2	lipofectamina	13	0/13	0/13
yCFIRES/Sat2	lipofectamina	16	3/16	2/3
yCFTR/Sat2	DOTAP	7	0/7	
yCFIRES/Sat2	DOTAP	8	0/8	
yCFTR/Sat2	PEI 25K	0		
yCFIRES/Sat2	PEI 25K	0		
Totale cloni MC1-CFTR				2



YAC purificati da gel

Costruzione di MC1-CFTR: strategia II

Fusione delle cellule CHO-MC1 con sferoplasti di lievito
(yCFTR e yCFIRES)

2x10⁶ cellule CHO-MC1 trasfettate con Sat2

+

108 protoplasti contenenti YAC-CFTR



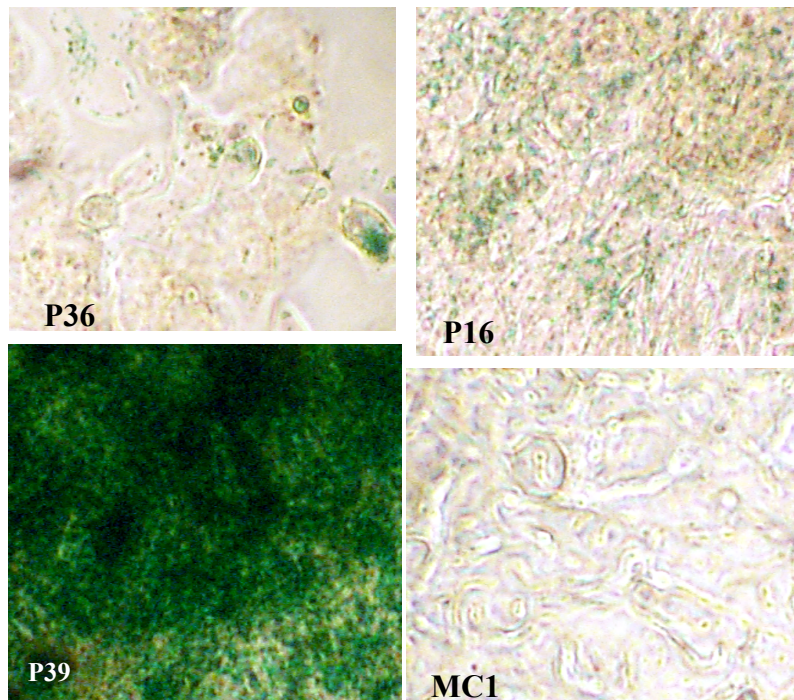
Fusione con PEG

Selezione per G418 e analisi dei cloni

Cloni MC1-CFTR ottenuti mediante fusione con gli sferoplasti

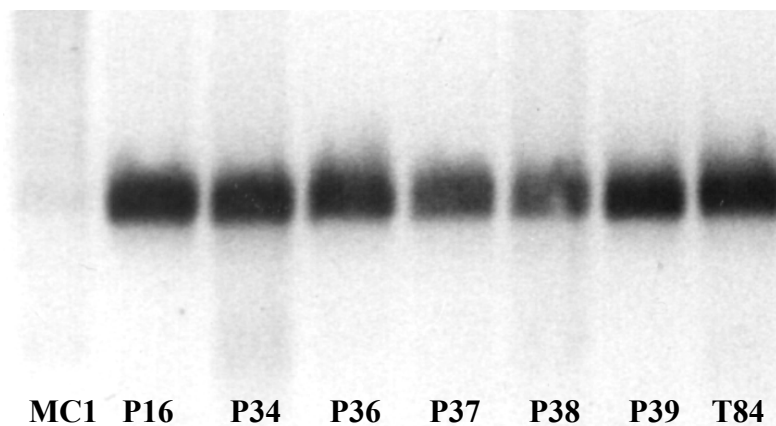
YAC	Cloni neoR	LacZ pos	cloni CFTR	MC1-CFTR
yCFTR	40	nd	nd	nd
yCFIRES	30	6/30	6/30	4/6
Totale cloni MC1-CFTR				4

Analisi dei cloni P mediante saggio della beta-galattosidasi



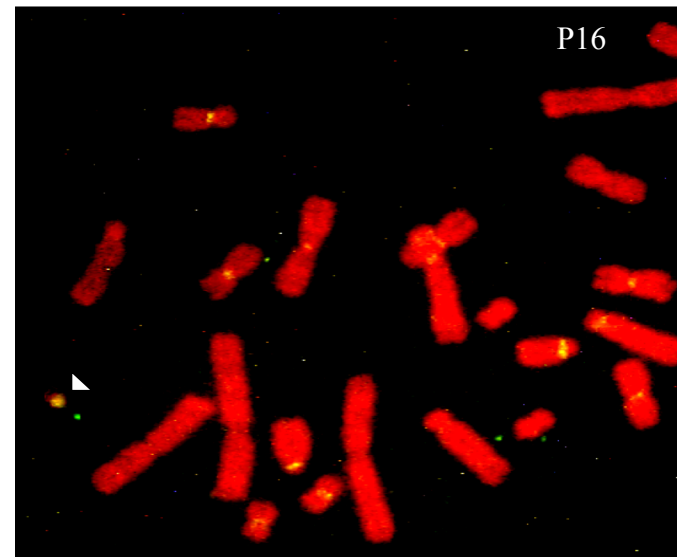
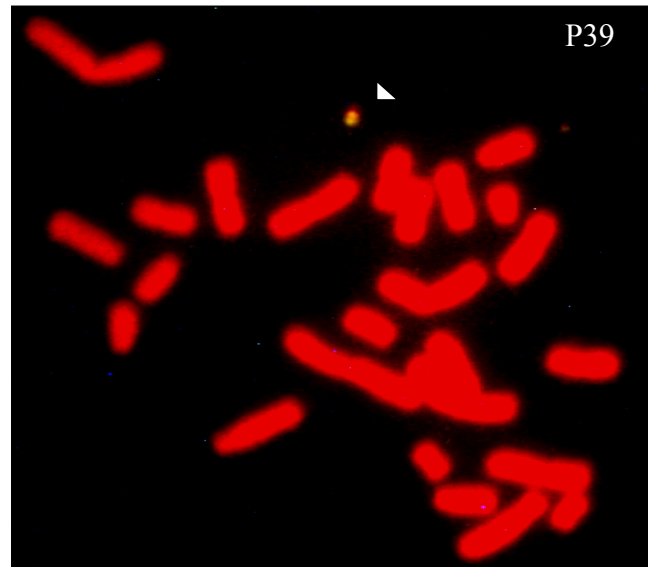
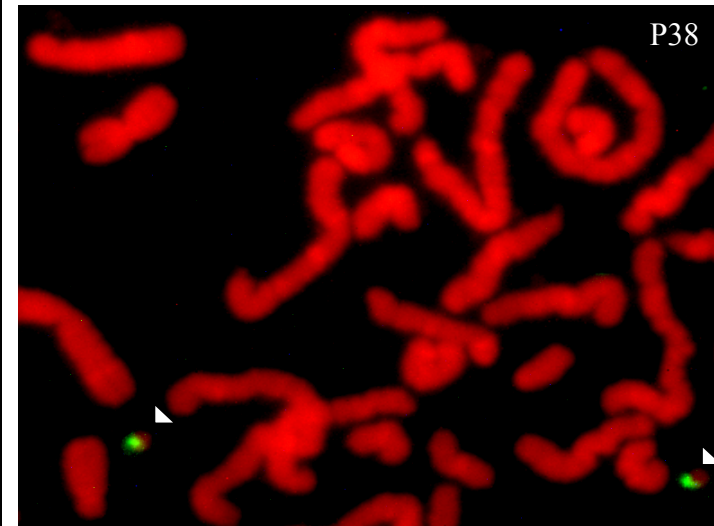
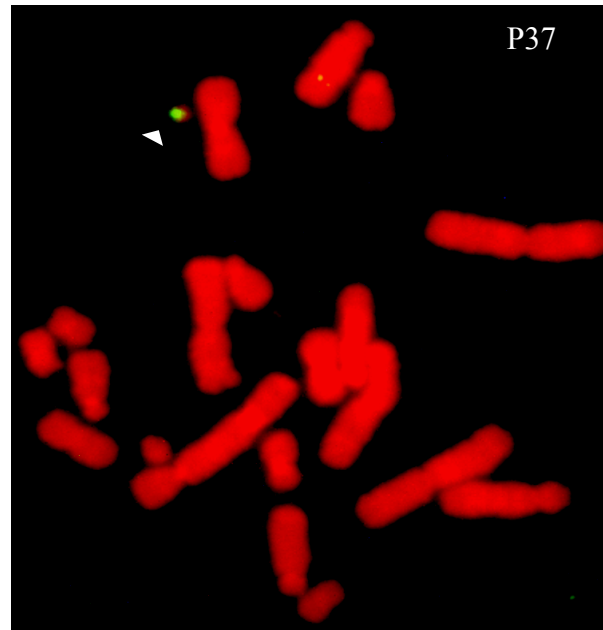
cloni	attività β -gal
P16	**
P34	*
P36	*
P37	*****
P38	*****
P39	*****
MC1	negativo

PCR esone 10 del CFTR

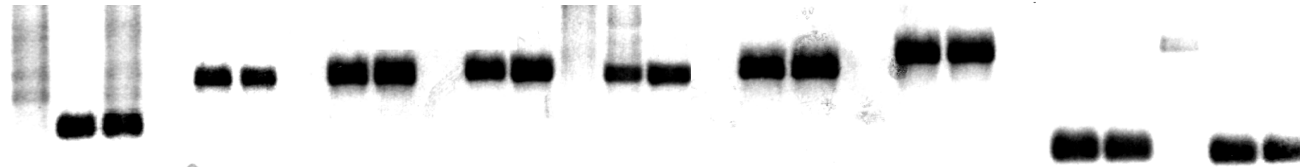


Identificazione dei cloni P contenenti MC1-CFTR mediante FISH

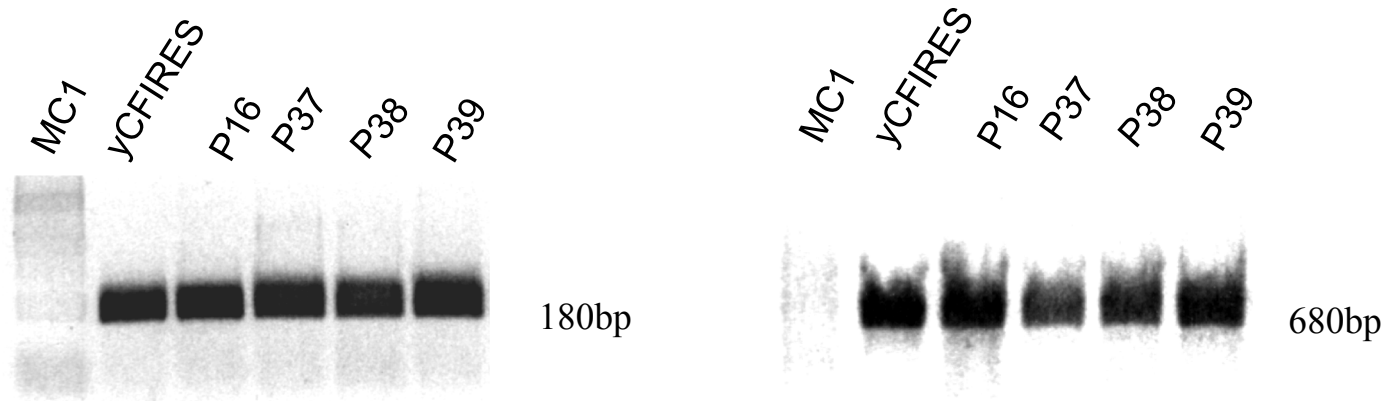
(Avidina-FITC)



Exon PCR



Ura3 e Amp-ori PCR



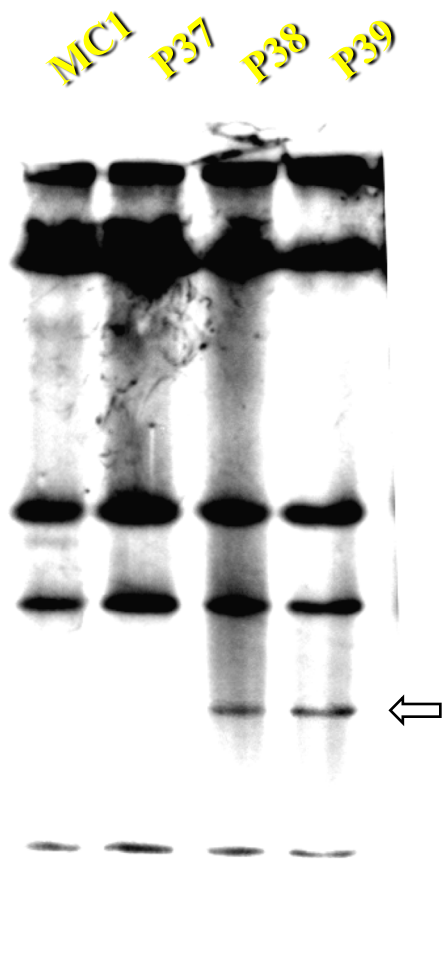
braccio destro URA3

braccio sinistro Amp-ori

Stabilità mitotica di MC1-CFTR nei cloni P

Stabilità mitotica di MC1-CFTR				
CLONE	30g + G418	30g – G418	60g + G418	60g – G418
P37	92/100	92/100	93/100	92/100
P38	91/100	92/100	91/100	93/100
P39	93/100	94/100	96/100	95/100

Stabilità molecolare di MC1-CFTR

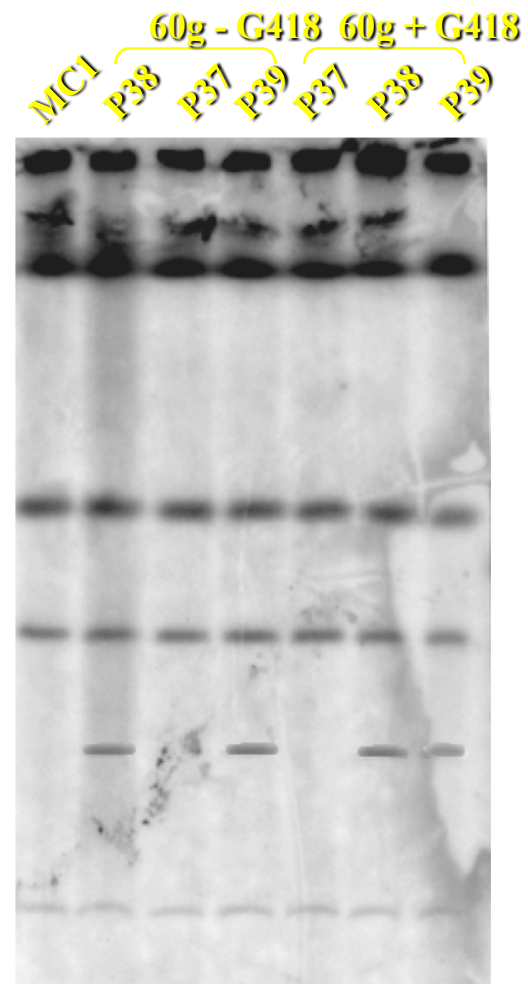


Sonde

Sat2



Alfoide



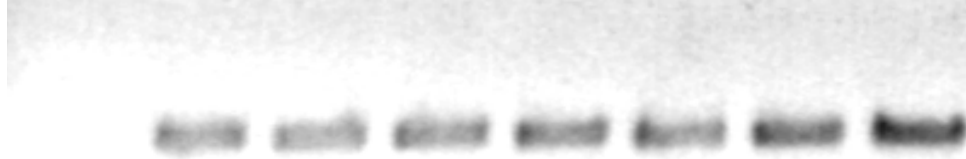
Sat2

Analisi dell'espressione del CFTR nei cloni contenenti MC1-CFTR

RT-PCR

MC1 T84 P16 P34 P36 P37 P38 P39

Esoni 7-9



Esoni 11-13



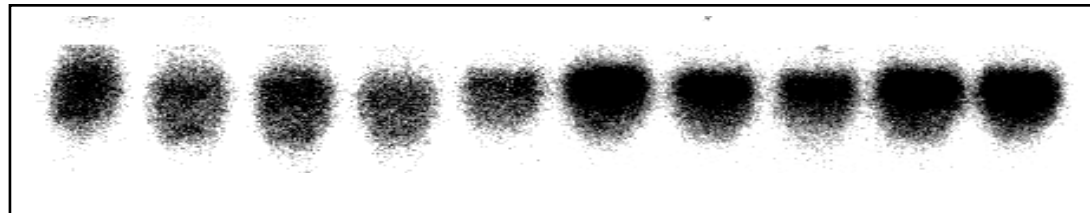
Northern

T84 CHO CHO-MC1 L1 P16 P34 P36 P37 P38 P39

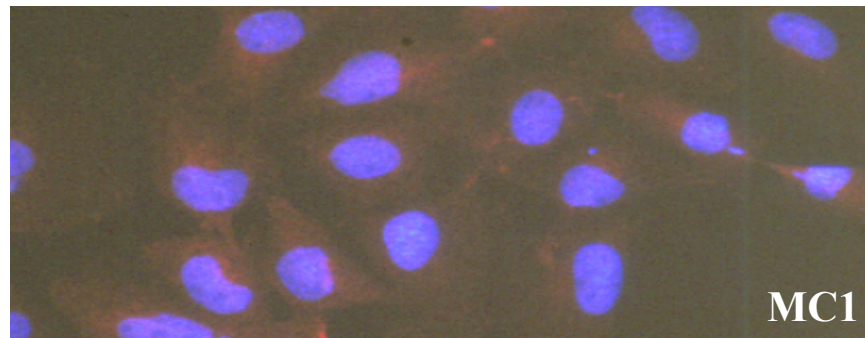
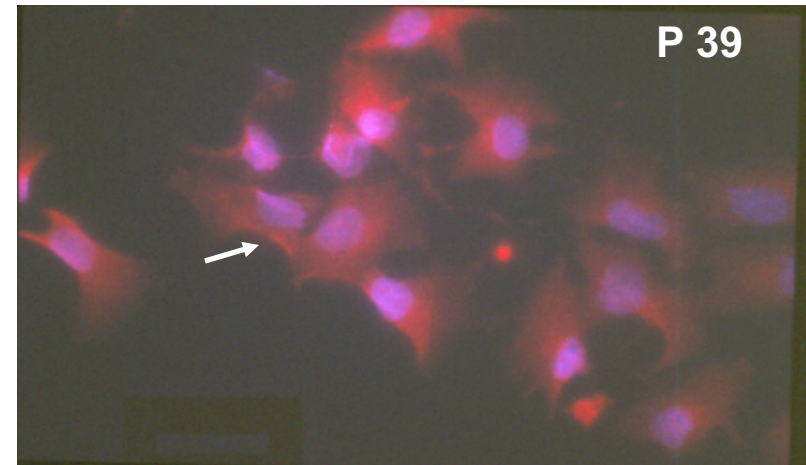
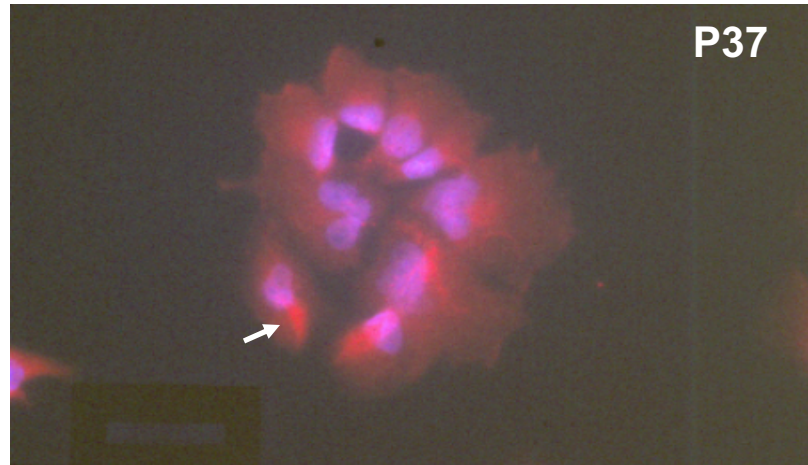
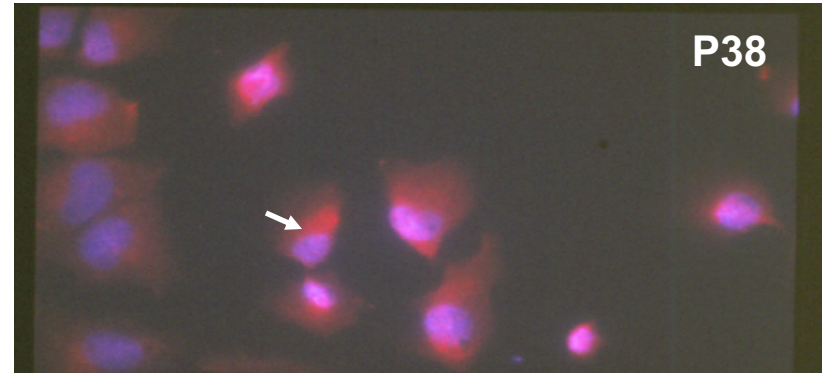
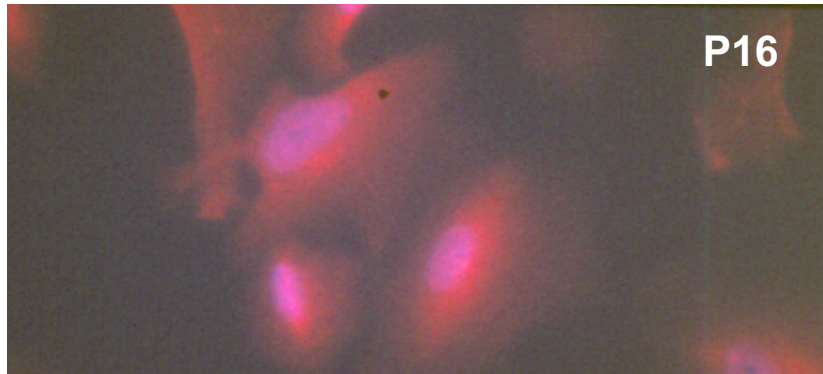
CFTR



Actina



Localizzazione mediante immunofluorescenza della proteina CFTR



L' anticorpo monoclonale utilizzato per rilevare la proteina CFTR è MATG 1031 (diluizione 1:100)

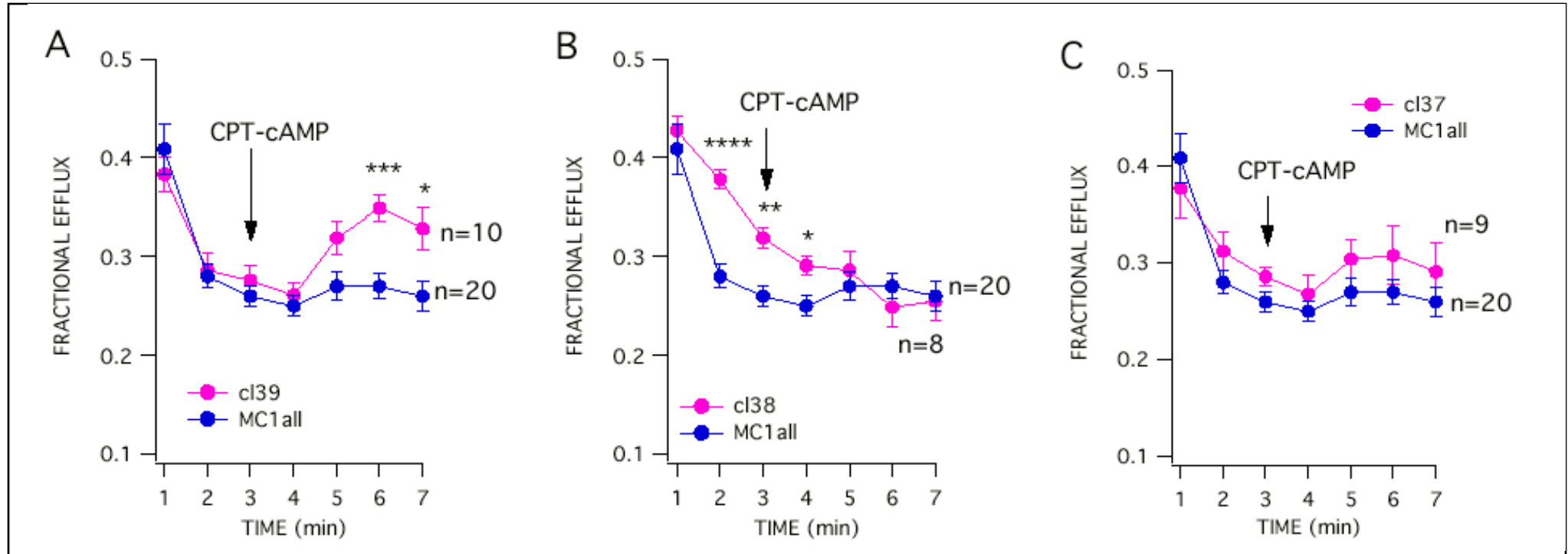
(Transgene, Strasbourg, France)

Dott. M. Conese

Laboratorio DIBIT San Raffele, Mi

Misurazione dell' efflusso di Cl^- nei cloni contenenti MC1-CFTR

Dott. O. Zegarra
Istituto G. Gaslini, Ge



CONCLUSIONI:

- è stato introdotto l'intero gene umano CFTR in MC1
- l'introduzione del gene esogeno non ha alterato la funzionalità del minicromosoma
- il gene CFTR introdotto in MC1 viene attivamente trascritto
- la proteina prodotta da MC1-CFTR viene correttamente processata e forma un canale attivo

PROSPETTIVE FUTURE:

- trasferimento di MC1-CFTR in modelli FC, sia in vitro che in vivo, ed analisi degli effetti terapeutici
- riduzione delle dimensioni di MC1, senza alterarne le proprietà funzionali
- sviluppo di nuovi sistemi di trasferimento, oltre al trasferimento cromosomico mediato dalle microcellule