

Sistemi d'espressione mediante in vitro transcription/translation

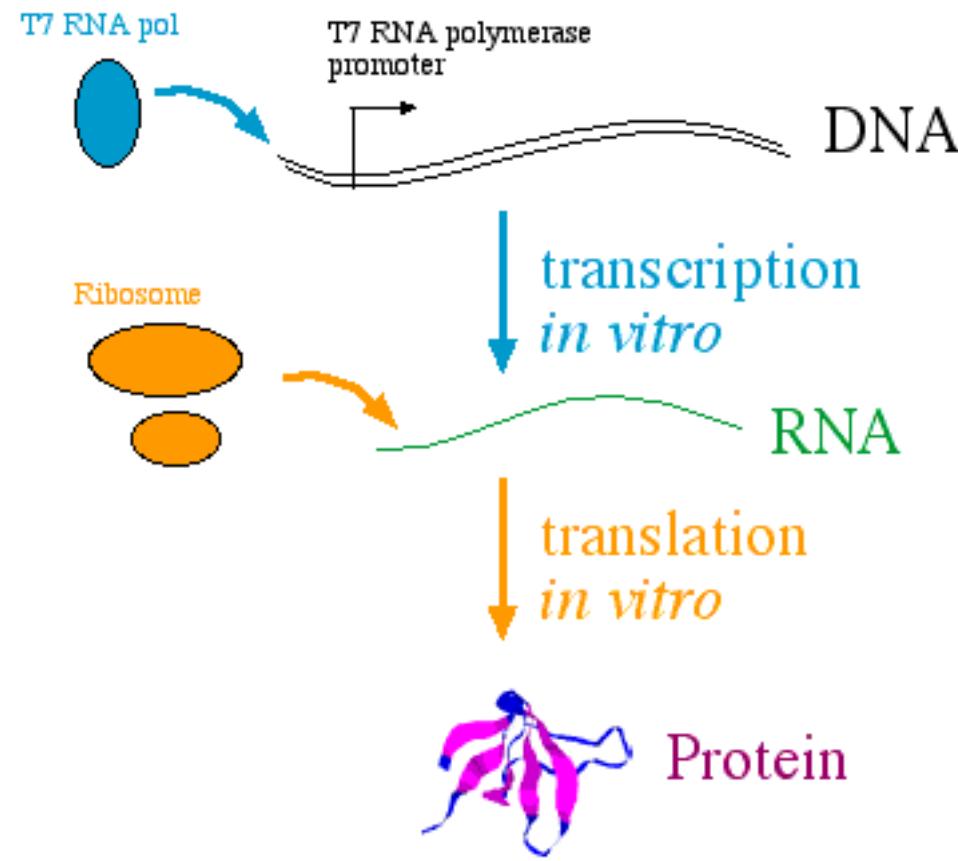
-Sistemi d'espressione mediante small-scale transfection in mammalian cells

Attraverso queste tecniche si hanno ridotte concentrazioni
di proteine esogene

La qualità di queste proteine è però molto elevata

Entrambi gli approcci sono indicati per confermare funzioni
specifiche (DNA-binding activity, transcriptional regulation).

Sistemi d'espressione mediante in vitro transcription/translation





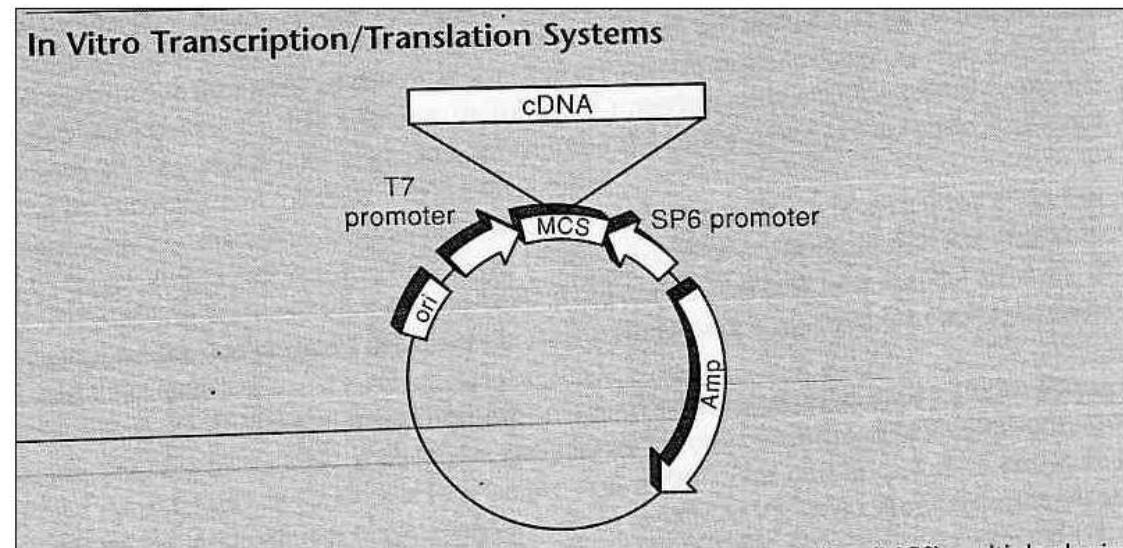
Sistemi d'espressione mediante in vitro transcription/translation

- promotori di origine fagica (T7,T3,SP6)
- RNA polimerasi di origine fagica
- mRNA stabilizzati nella struttura mediante modificazione (mRNA capped o aggiunte di sequenze al 3' nontradotte UTR)



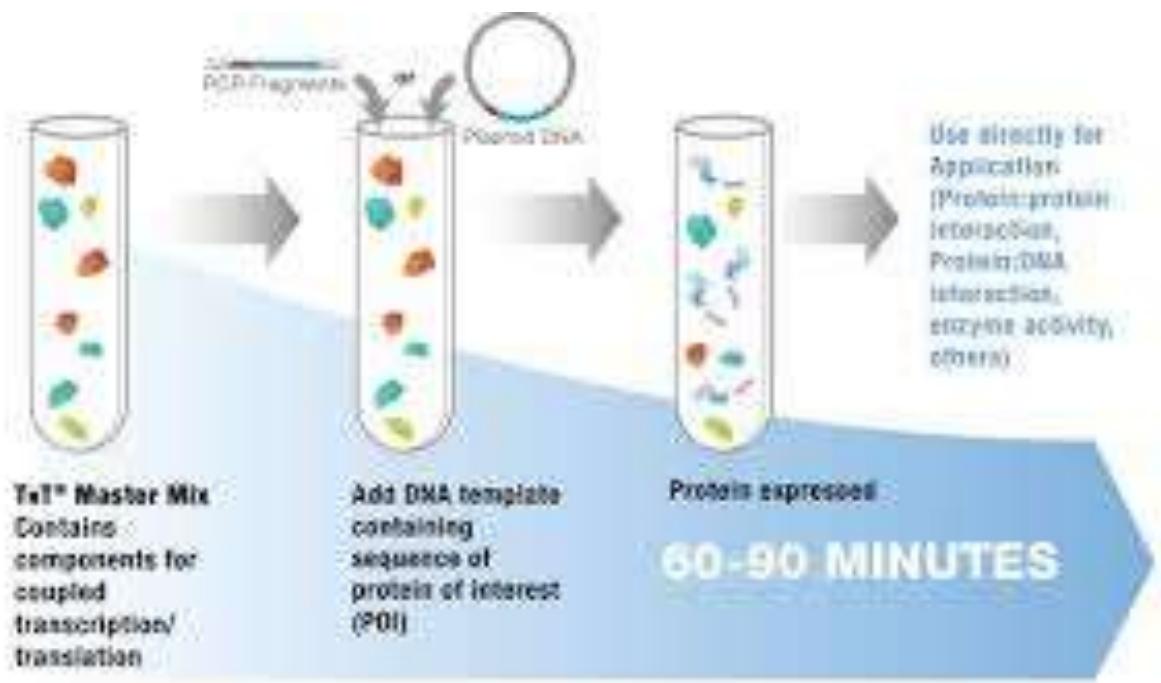
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Visualizzatore immagini e fax per Windows

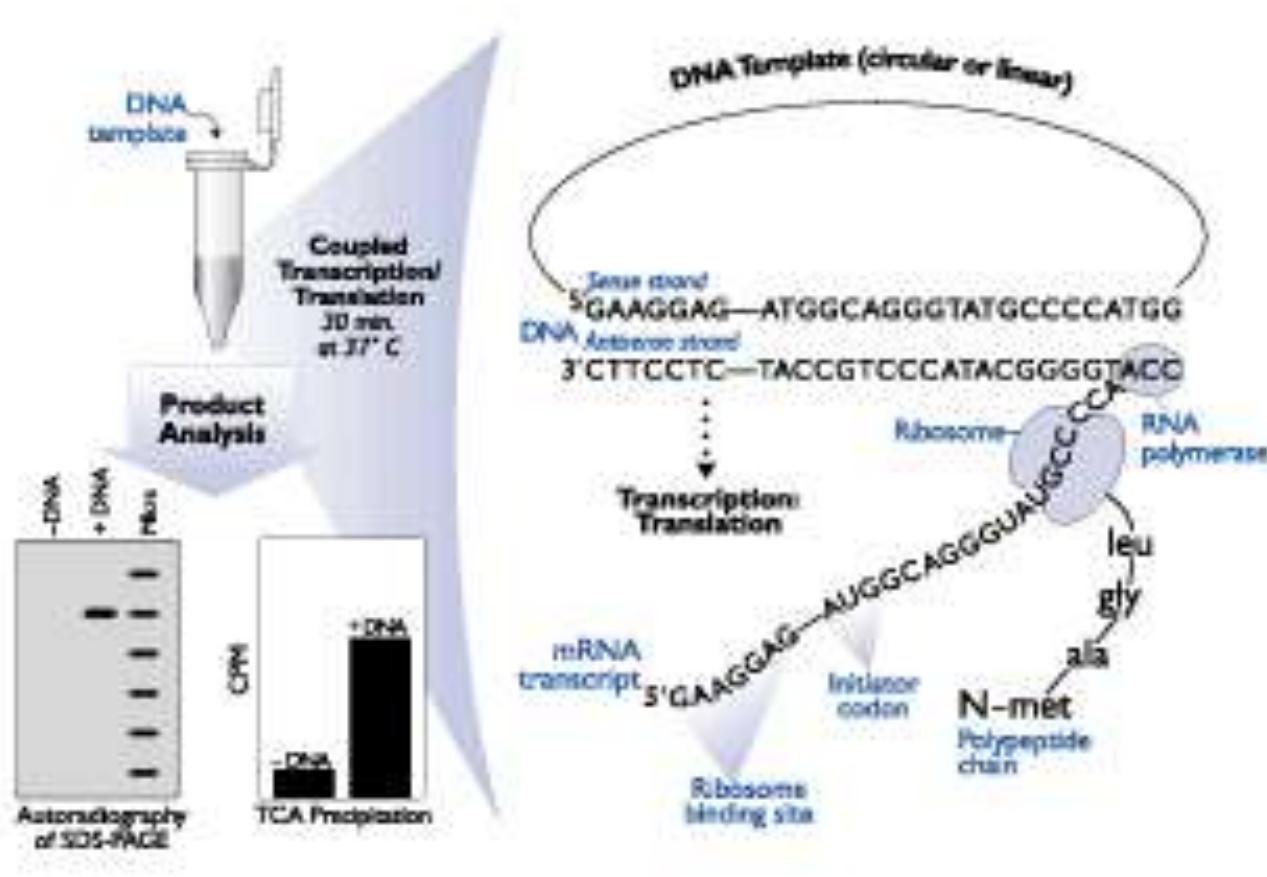




Sistemi d'espressione mediante in vitro transcription/translation



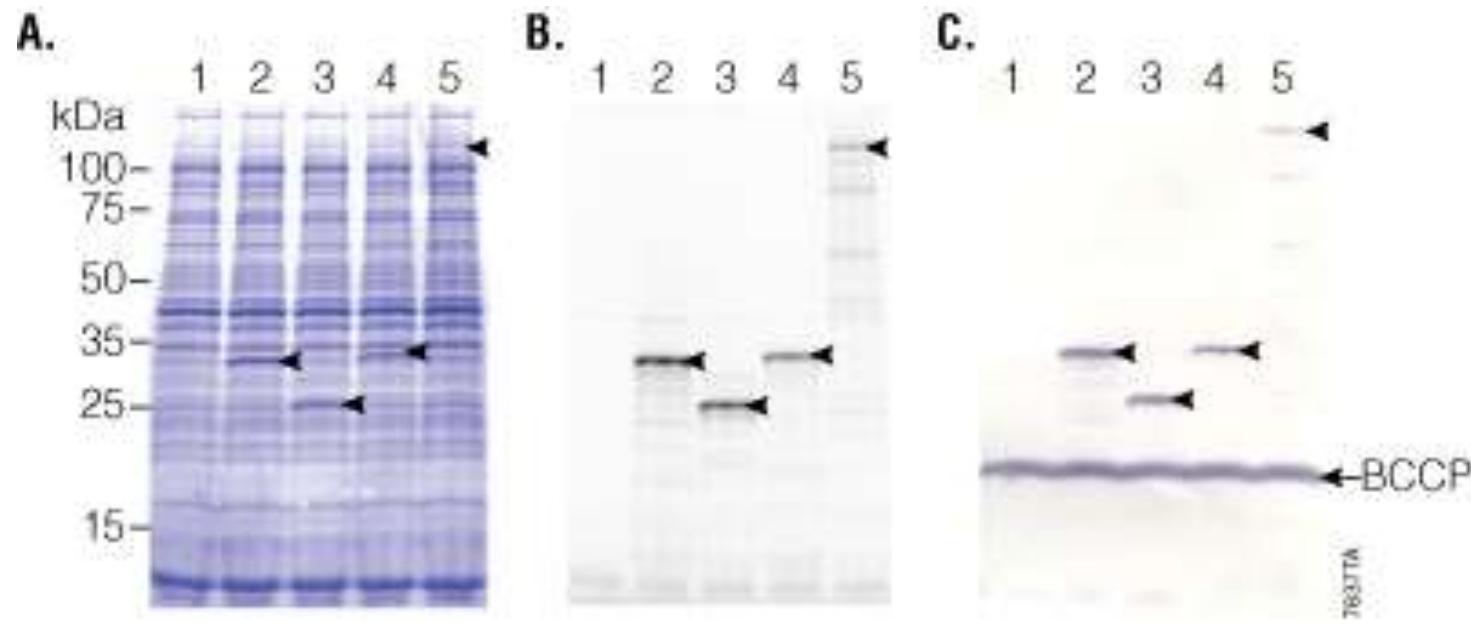
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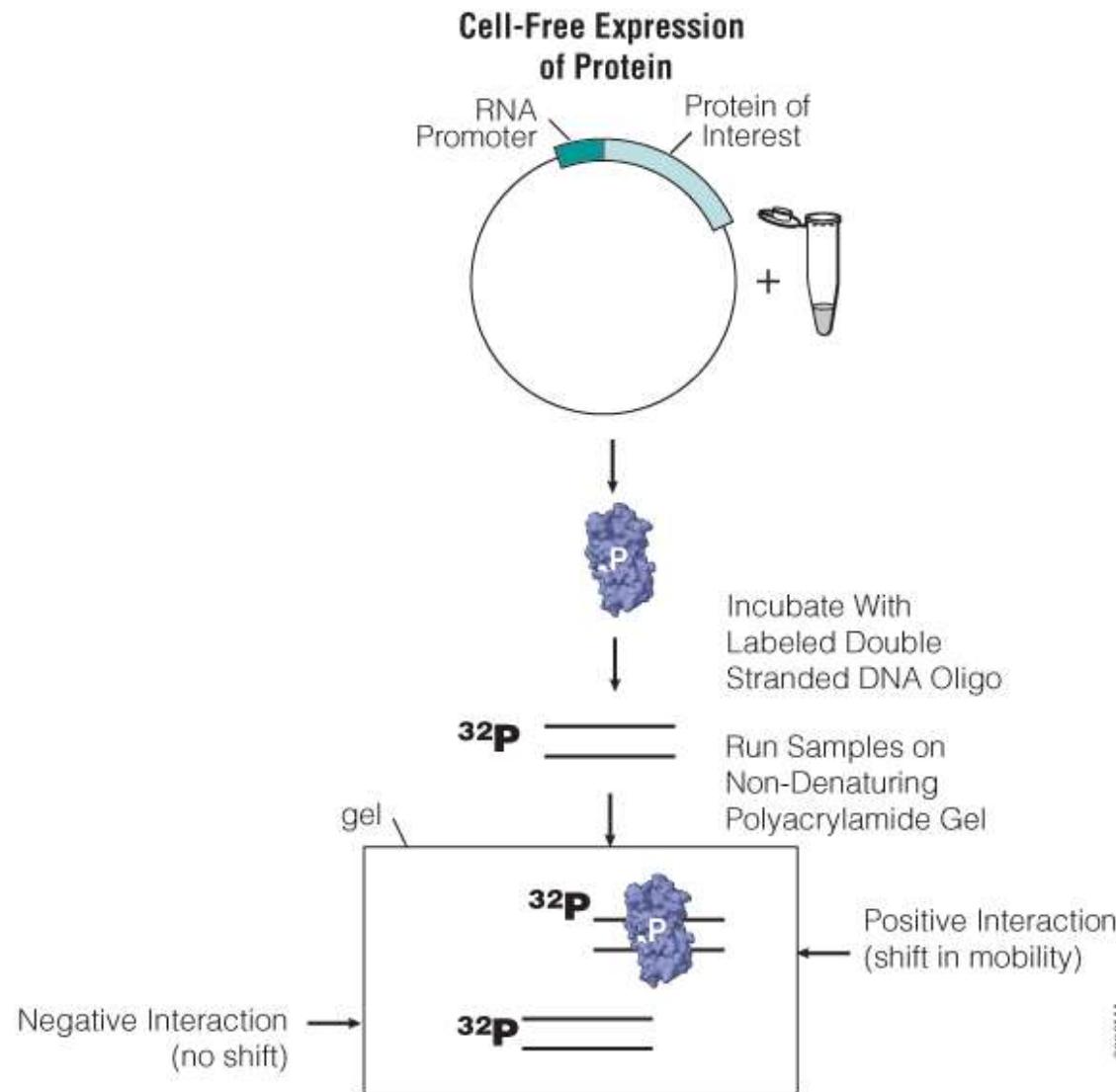
Sistemi d'espressione mediante in vitro transcription/translation

Analisi di proteine mutate



Sistemi d'espressione mediante in vitro transcription/translation

Analisi di una DNA binding protein mediante EMSA



Vantaggi dei sistemi in vitro transcription/translation

- produzione di piccole quantità di proteina
- estrema facilità (kit commerciali)
- espressione di proteine di piccola e grossa taglia

Svantaggi dei sistemi in vitro transcription/translation

- basse rese
- contaminazioni da altre proteine presenti negli estratti totali usati nella traduzione
- proteine tronche o mutate

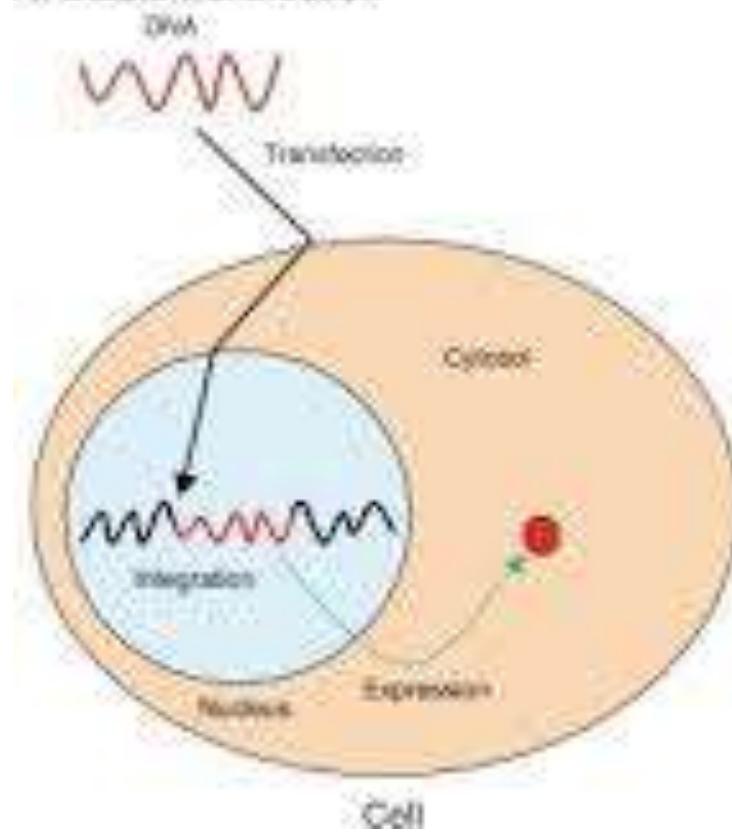
Sistemi d'espressione mediante small-scale transfection in mammalian cells

- transfezione transiente
- transfezione stabile
- espressione costitutiva
- espressione inducibile

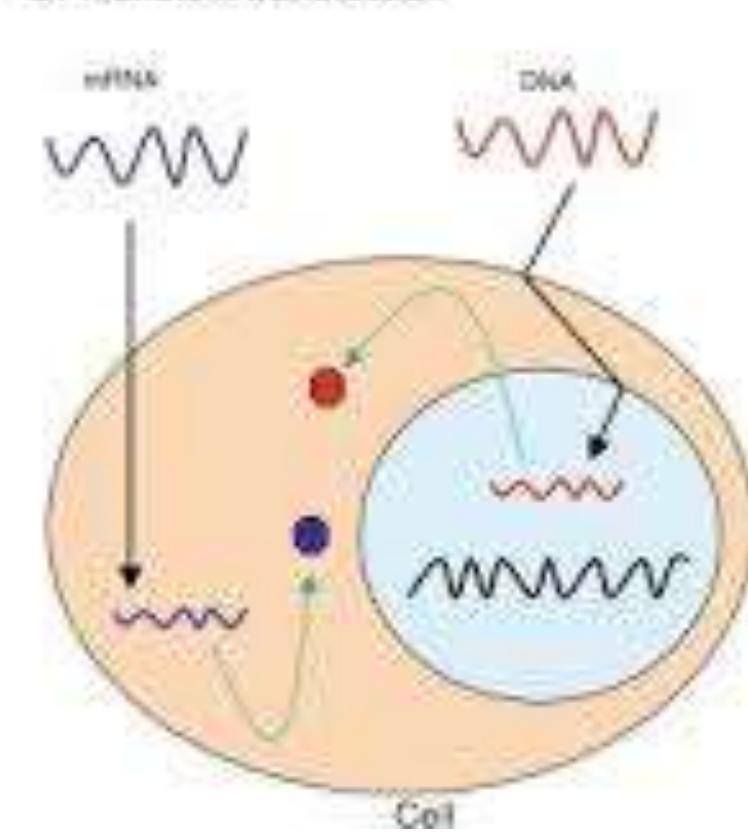
ZNF224 repression activity

Stable or transient ?

A. Stable transfection



B. Transient transfection

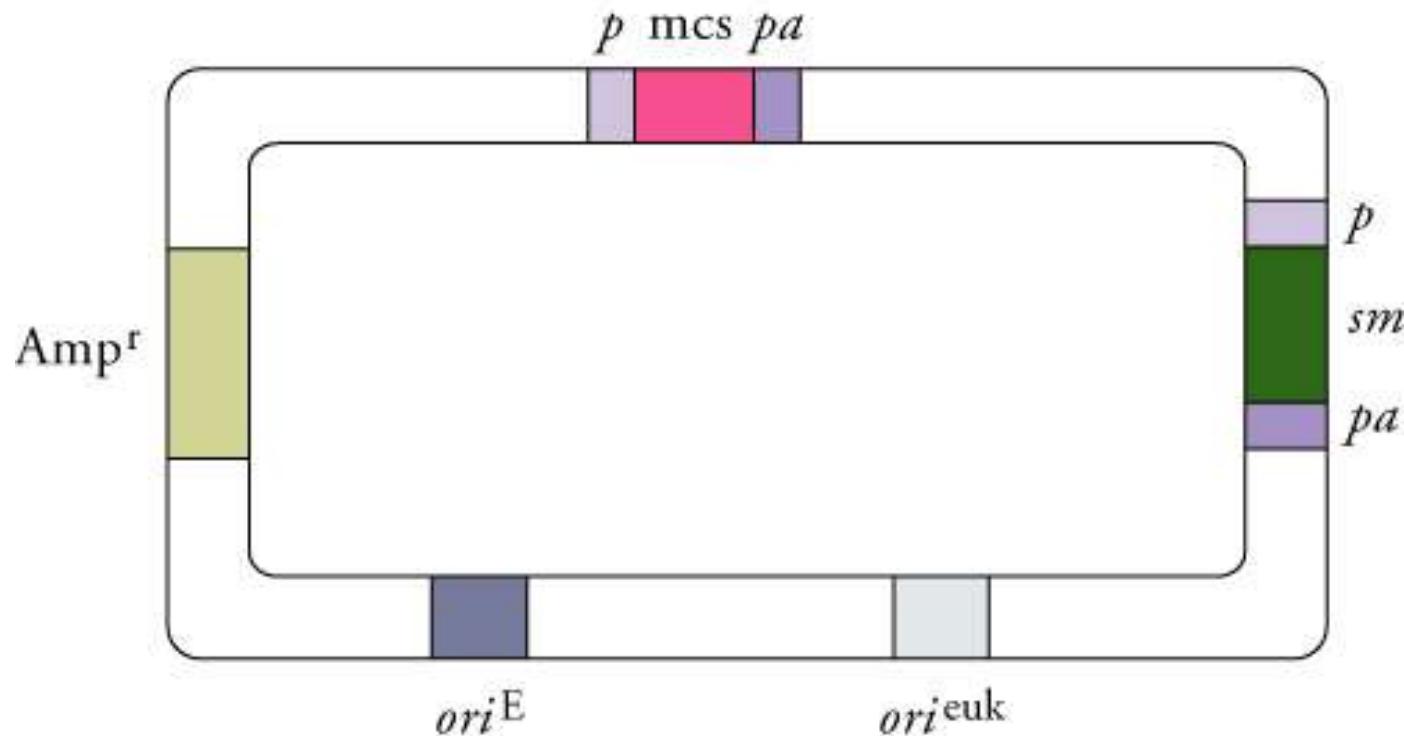


Caratteristiche dell'espressione mediante small-scale transfection

- plasmidi d'espressione per cellule di mammifero
- cellule recipienti quali COS7 o 293T
- promotori con sequenze “enhancer” di origine virale (CMV, SV40,RSV)

Sistemi d'espressione mediante small-scale transfection in mammalian cells

Un tipico vettore d'espressione eucariotico





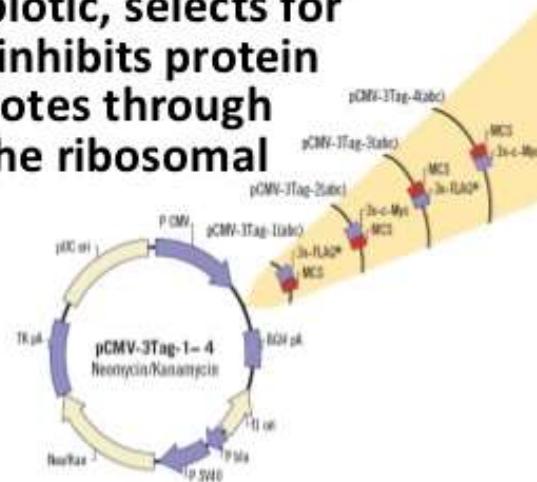
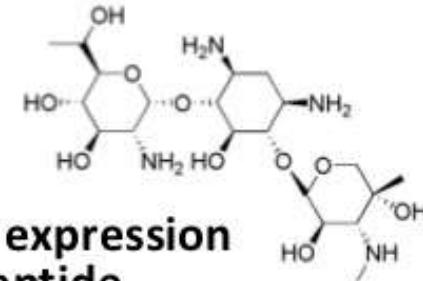
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Antibiotics Resistance as Selectable markers

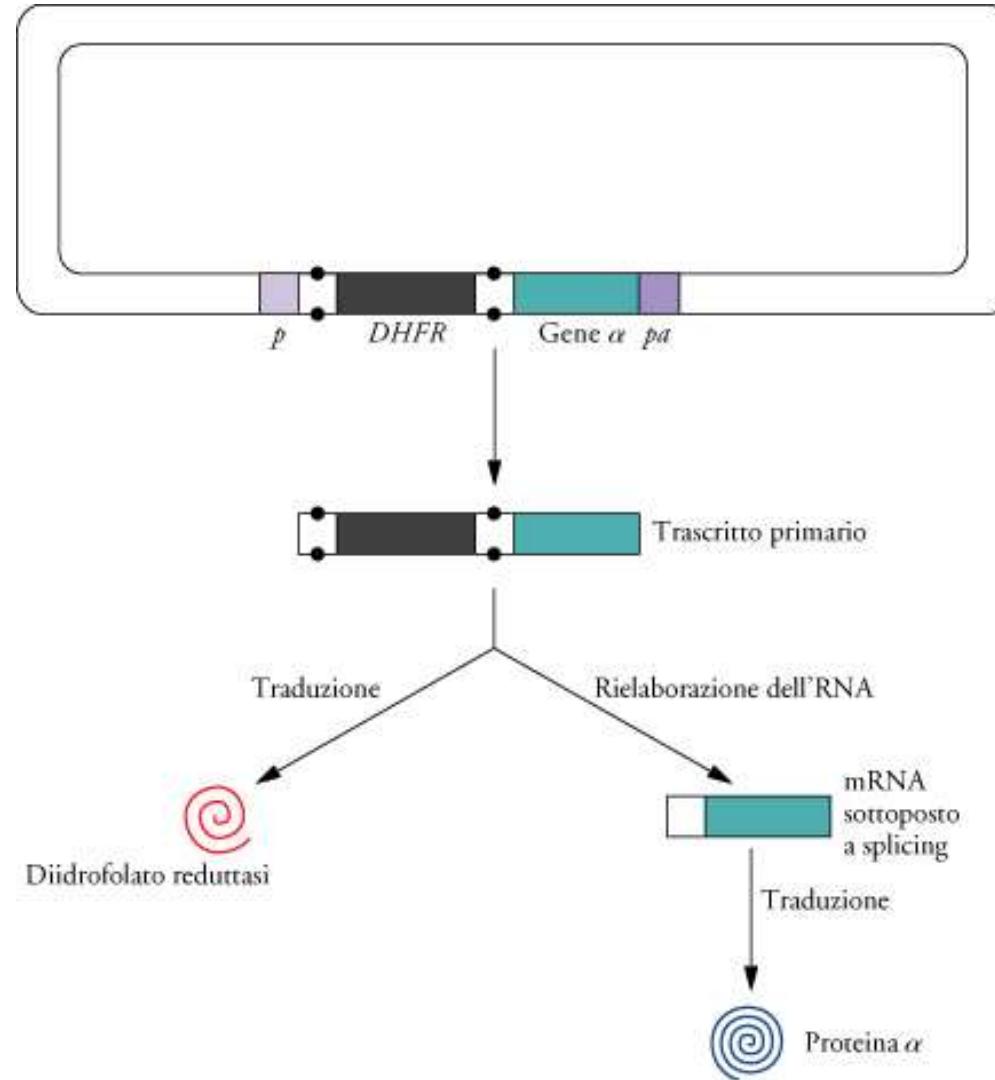
Selectable marker gene(s)	Resistance against	Uses
Neomycin phosphotransferase II (npt II)	G418 sulphate, Kanamycin, Neomycin, etc	Dicots/ monocots
Hygromycin phosphotransferase (hpt or aphiV)	Hygromycin B	Dicots/monocots
Aminoglycoside -3 adenyltransferase (<i>aad A</i>) or streptomycin resistance gene (<i>spt</i>)	Streptomycin/ Spectinomycin	dicots
Gentamicin 3-N- acetyl transferase (<i>gat</i>)	Gentamicin	Dicots
Dihydrofolate reductase (<i>dfr</i>)	Methotrexate	Dicots
Unknown gene (<i>ble</i>) of Tn5 transposon	Bleomycin	

Sistemi d'espressione mediante small-scale transfection in mammalian cells

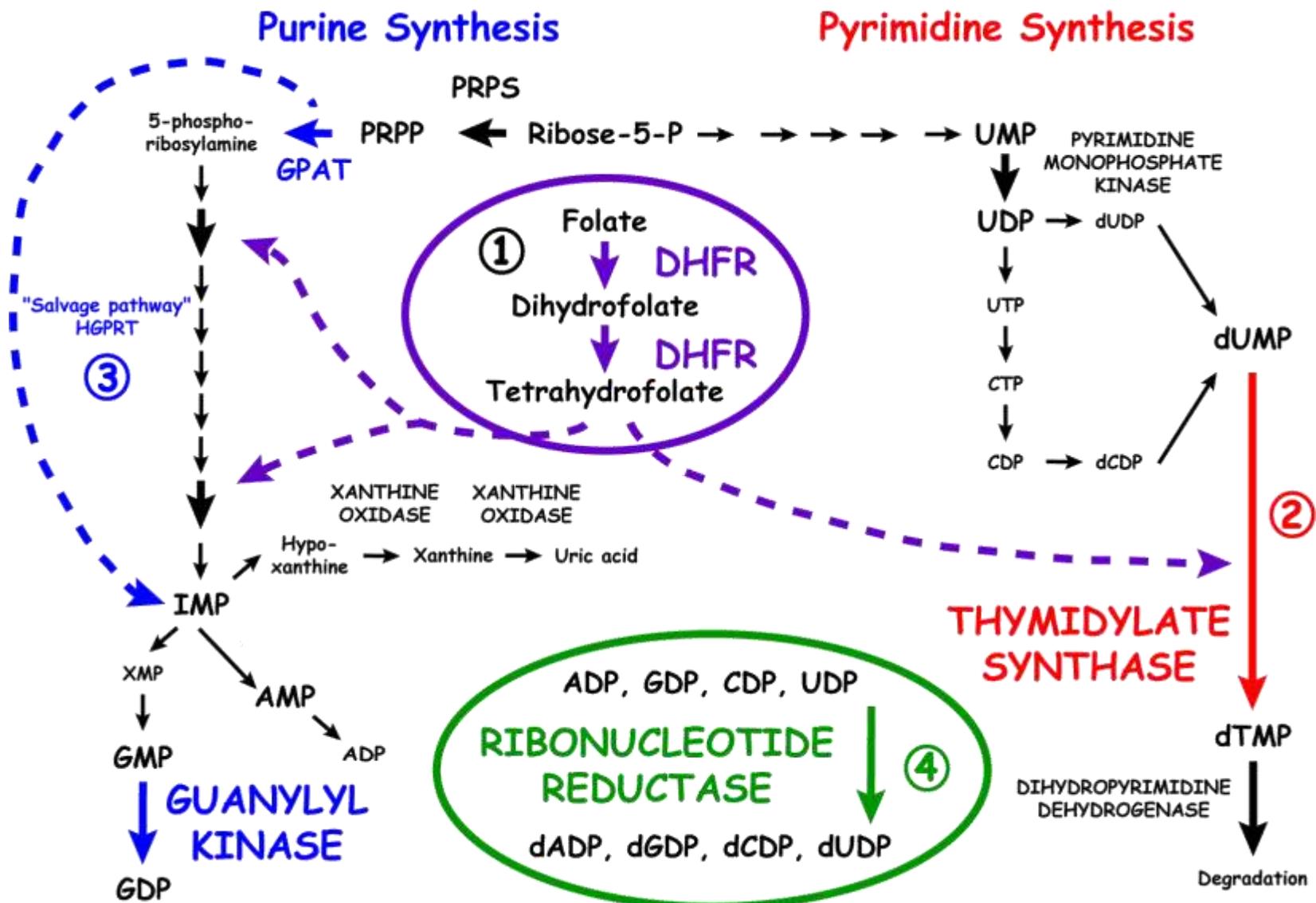
- G418 is an aminoglycoside antibiotic, selects for expression of neomycin-resistant genes. G418 blocks polypeptide synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells.
- Blasticidin S is a peptidyl nucleoside antibiotic, selects for expression of the bsr gene. It specifically inhibits protein synthesis in both prokaryotes and eukaryotes through inhibition of peptide-bond formation in the ribosomal machinery.



Sistemi d'espressione mediante small-scale transfection in mammalian cells

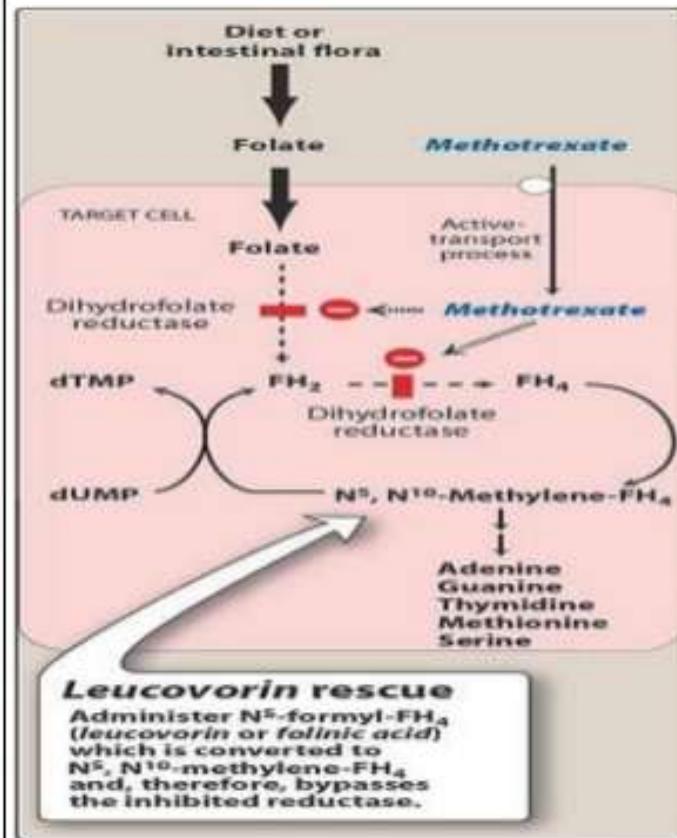


Sistemi d'espressione mediante small-scale transfection in mammalian cells



Sistemi d'espressione mediante small-scale transfection in mammalian cells

16.1 MECHANISM OF ACTION OF METHOTREXATE



- **Methotrexate** potently inhibits Dihydrofolate reductase (DHFR).
- This leads to decreased production of compounds adenine, guanine and thymidine and the amino acids methionine and serine, depletion of thymidine.
- Finally depressed DNA, RNA, and protein synthesis and, ultimately, to cell death.

FH₂ = dihydrofolate; FH₄ = tetrahydrofolate; dTMP = deoxythymidine monophosphate; dUMP = deoxyuridine mono phosphate.

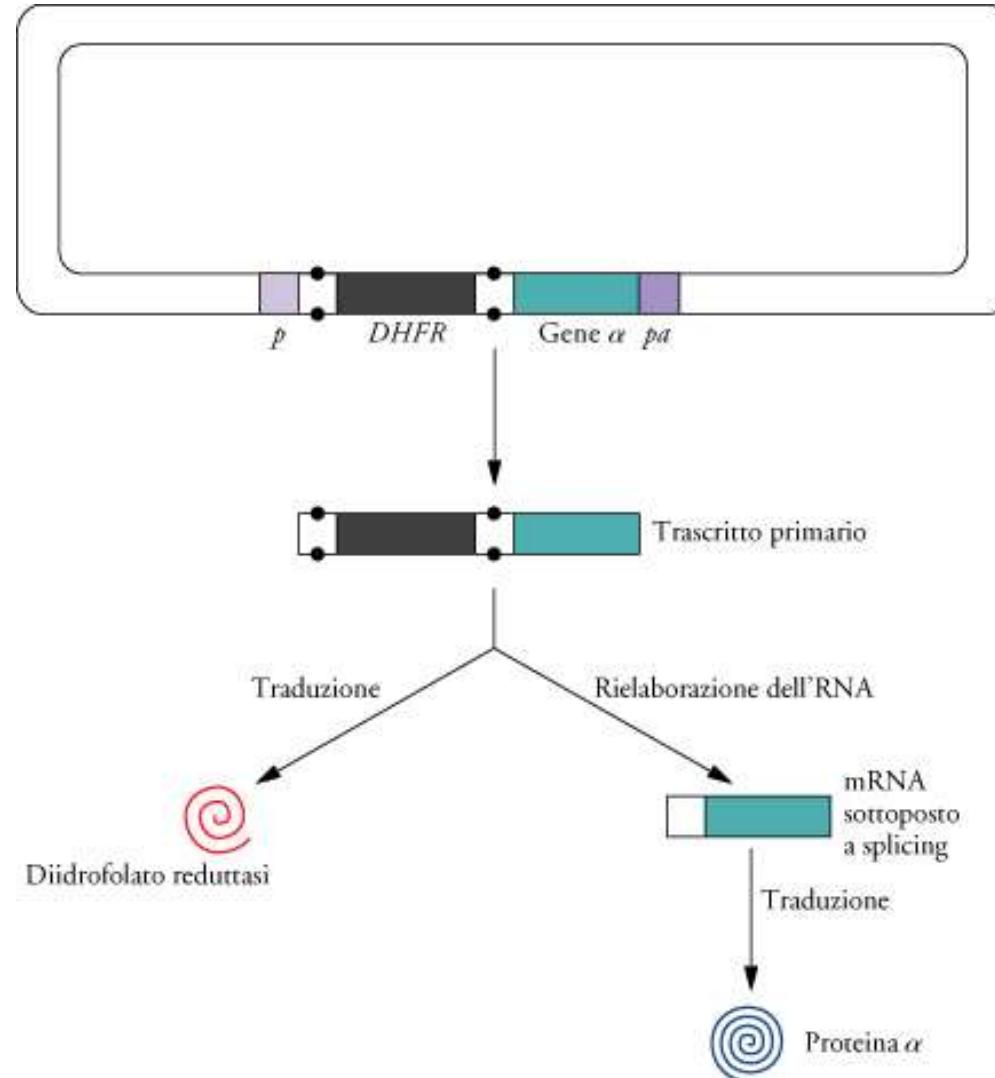


Sistemi d'espressione mediante small-scale transfection in mammalian cells

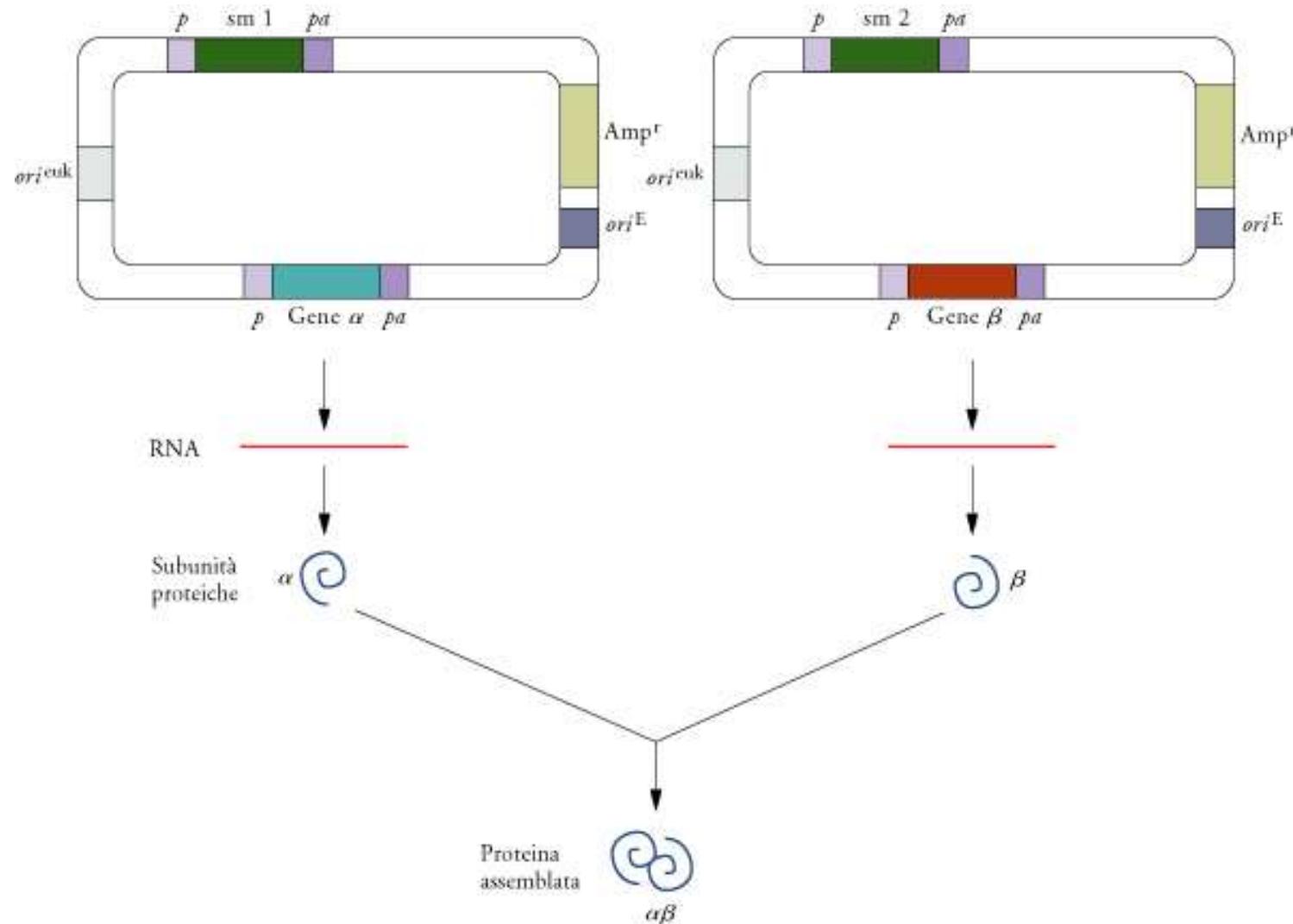
Mechanism of Action of Methotrexate

- Has higher affinity for DHFR than dihydrofolic acid
- Binds to DHFR
- Inhibits DHFR enzymatic activity
- Dihydrofolic acids are not reduced to tetrahydrofolates
- Purine and pyrimidine synthesis will not happen
- One-carbon transfer reactions require specific coenzymes synthesized from tetrahydrofolates
- Inhibit DNA and protein synthesis
- Cells are not dividing

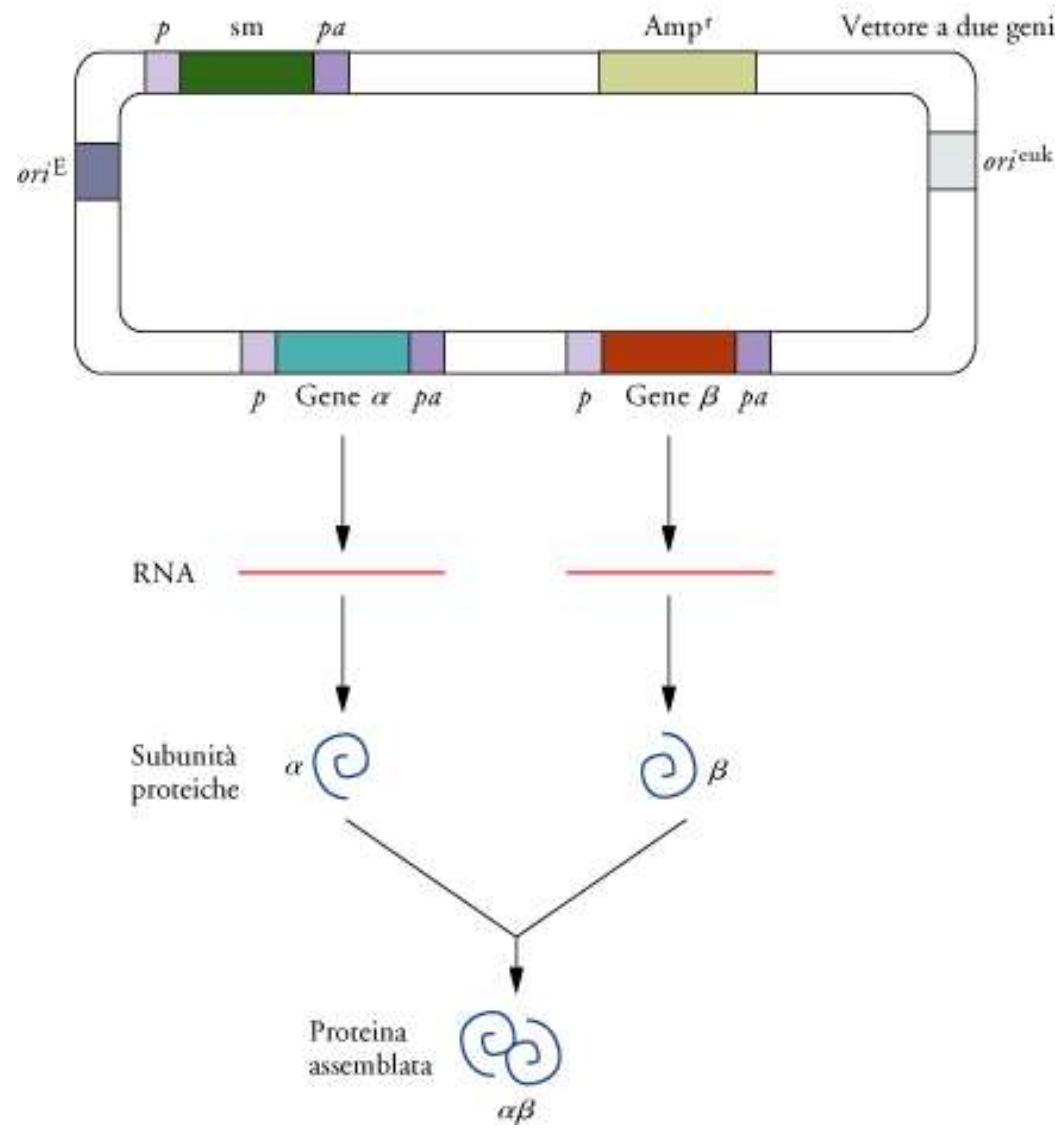
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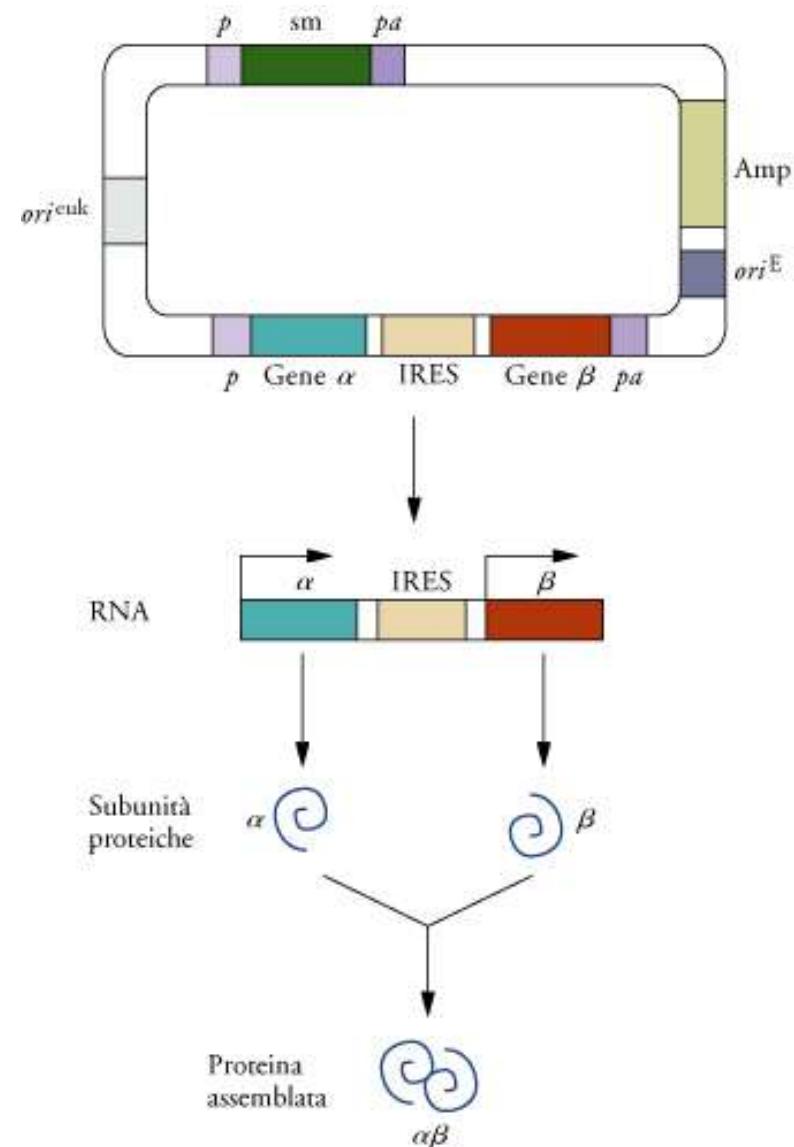
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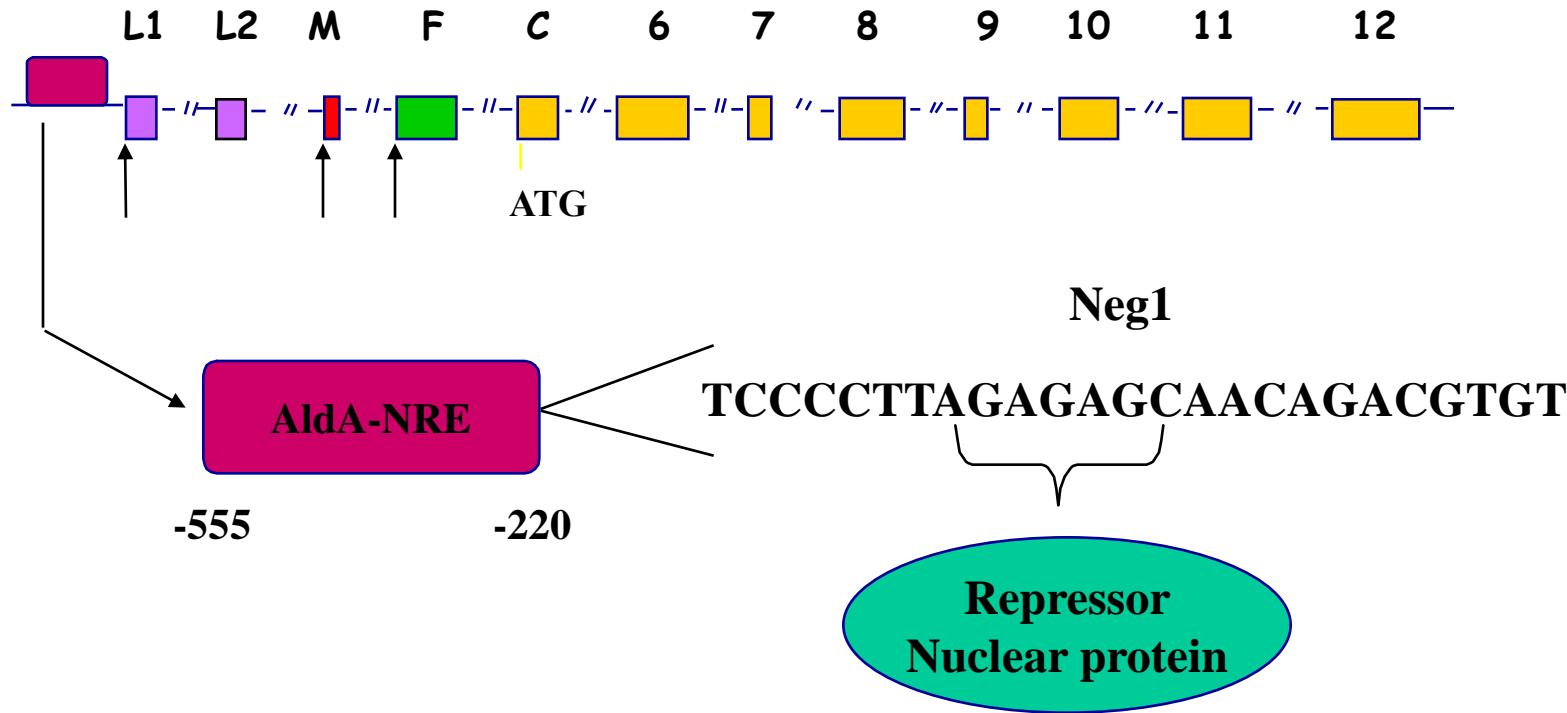
Sistemi d'espressione mediante small-scale transfection in mammalian cells



Sistemi d'espressione mediante small-scale transfection in mammalian cells



AldA-NRE / ZNF224 interaction





ZNF224 cDNA sequence

1 GAGTCCAAACATTGGAGTCGGACACTTCCGCTCGGGACTGAGGTTGCTGCAGTTTCCGCGATAGTTG
 101 CCTTCTGAATTCTGGACCTACGCATTGGATCCTCAAAGAACTGCTGAATACCACTAGAAACATACTGTAACCAG
 201 CAGCAAGGAAGCCCACGTACCAAGGGGCTGCTTGGCACAACTCTGCTTCCAGGAACCTGCATCACTCAGGACTCTG
 301 AAATGACCACGTTCAAGGAGGCAATGACCACTCAAGGACGTTGCTGGGTCTCACTGAGGAAGAGCTGGGGCTGCTG
 M T T F K E A M T F K D V A V V F T E E E L G L L
 401 TCGAGATGTGATGCTGGAGAACCTCAGGAACCTGCTCTCAGTGGACATCAAGCATTCCACAGGGATACTTCCACT
 R D V M L E N F R N L L S V G H Q A F H R D T F H I
 501 ATGATGAAGACAGCAATCCAAGGGAAAGGAATTCAAGGAGACAAGATCCAACACTGAGATGGAGACTGTTCAGAACGC
 M M K T A I Q R E G N S G D K I Q T E M E T V S E A
 601 TCCAGCAAATCTGGGAAAAAAATTGCAAGTGATTIAACCAGGTCTCAAGAAGACTGGTGATAAAATAGCTCTCAGTTCTCC
 Q Q I W E K I A S D L T R S Q D L V I N S S S Q F S
 701 GACTGAGGCAGGACTATCTGTAATTCACACAAGACAGAAATCTCCAGGGCAATGGATATAAACCATCCTTCAGTG
 T E A G L S V I H T R Q K S S Q G N G Y K P S F S I
 801 CAACAATTACACTCAGGAGAGAAATCTCATACGTGTGATGAGTGTGGAAAGAACCTTTGTTACATCTCAGGCCCTTCG
 Q Q L H S G E K S H T C D E C G K N F C Y I S A L R
 901 GAGAGAAAATGCTATAAGTGTGACGTGTGGTAAGGAATTCAAGTCAGAGTTCACATCTGCAAACATCATCAGAGAGTC
 E K C Y K C D V C G K E F S Q S S H I L Q T H Q R V
 1001 ATGTGTGGAATGTGGGAAAGGCTTCAGTCGTAGATCAGCACTTAATGTTCATCACAAATTACACACAGGAGAGAAC
 C V E C G K G F S R S A L N V H H K L H T G E K I
 1101 AAGGCCTTCATTCAACGATTCCAGCTCAAGAACATCAGAGAACATCCATACGGGGAGAACGCCATTCAAATGTGATAT
 K A F I H D S O L O E H O R I H T G E K P F K C D I
 1201 GATCAAGACTTAATAGGCATTCCATGGTCACACGGCAGAGAACCATCCGATGTGATACGTGTGATAAGAGCTTT
 S R L N R H S M V H T A E K P F R C D T C D K S F
 1301 TCATCGCATGATCCACACAGGAGAGAACCATACAAATGTGAGGAGTGTGGAAAAGGCTTATTGTAGGCAGAGAC
 H E M I H T G E K P Y K C E E C G K G F I C R R D I
 1401 ACGGGAGAAAAGCCATATAATTGTAAGAGTGTGGGAAGAGCTTCAGATGGCCTCGTGTCTTGAAACATCAGCG
 T G E K P Y N C K E C G K S F R W A S C L L K H Q R
 1501 TCAAATGTGAAGAACATGTGGGAAGGGATTACACAAATTCAACATGCTATTCCACCCAGAGATCCCATAAGTGGAGAA
 K C E E C G K G F Y T N S Q C Y S H Q R S H S G E
 1601 TGGGAAGGGCTACAAAAGGAGGTTGGATCTTGACCTTCACAGCGCGTCATACAGGAGAGAACACTGTATAATTGTA
 G K G Y K R R L D L D F H Q R V H T G E K L Y N C I
 1701 CGGGCCCCATGTCTTTGAAACATGAGAGACTCCACAGTGGAGAAAACCATTCAAATGTGAAGAGTGTGGGAAGAG
 R A P C L L K H E B L H S G E K P F Q C E E C G K R
 1801 ATTCCCATCAGAGAGTTCACACTGGAGAAAAGCCATACAAATGTGAGAAGTGTGGAAAAGGGCTACAATAGTAAGTT
 S H Q R V H T G E K P Y K C E K C G K G Y N S K F
 1901 CCACACAGGAGAGAGACCATACAATTGTAAGGAATGTGGGAAGAGTTTGCGTGGCCTCGTGTCTTTGAAACATC
 H T G E R P Y N C K E C G K S F G W A S C L L K H C
 2001 CCTTTCAAATGTGAAGAGTGTGGAAAAGATTACTCAGAATTCAACAGCTTCATTCTCATCAAAGAGTGCACACTGG
 P F K C E E C G K R F T Q N S Q L H S H Q R V H T G
 2101 AGTGTGGGAAGGGCTTCAGCTGGCCCTCAACTCGTCTGACCCATCAGAGACGCCACAGCAGAGAACACCTCTCAA
 C G K G F S W S S T R L T H Q R R H S R E T P L K
 2201 TGTACAGAATTCTCATCAAAGTGCAGAGAAAAGTTCAAGTGTAGAAAAGCCATACAAATGTGAGGAGACTGTGGGA
 V O N S F S K V Q E K V H S V E K P Y K C E D C G I
 2301 CTTGATATGCATCAGAGGGTCCACATGGGAGAGAAAACATGGAAGTGTAGGGAGTGTGATATGTGCTTTAGTCAGGC
 L D M H Q R V H M G E K T W K C R E C D M C F S Q A
 2401 ATGTTCATGTTGGAGAAAACCTTAGTGTGATGGTGCAATAAGTCTTCACTCAGTCTTCATG 2466
 V H V G E K P *

Gli esperimenti di espressione in *E.coli* hanno dimostrato che :

- 1) la proteina espressa in *E.coli* attraverso la trascrizione e la traduzione è proprio la proteina che riconosce la sequenza specifica di repressione del gene di aldolasi A
- 2) la proteina KRAB-zinc finger ZNF224 è una DNA binding protein
- 3) il suo dominio di binding al DNA richiede che siano presenti almeno i primi 8 domini a dita di zinco
- 4) la proteina overespressa in *E.coli* è perfettamente funzionale
- 5) La proteina wild type e le isoforme mutanti per delezioni successive sono esattamente della taglia aspettata, come dimostrato dagli esperimenti di western blotting.

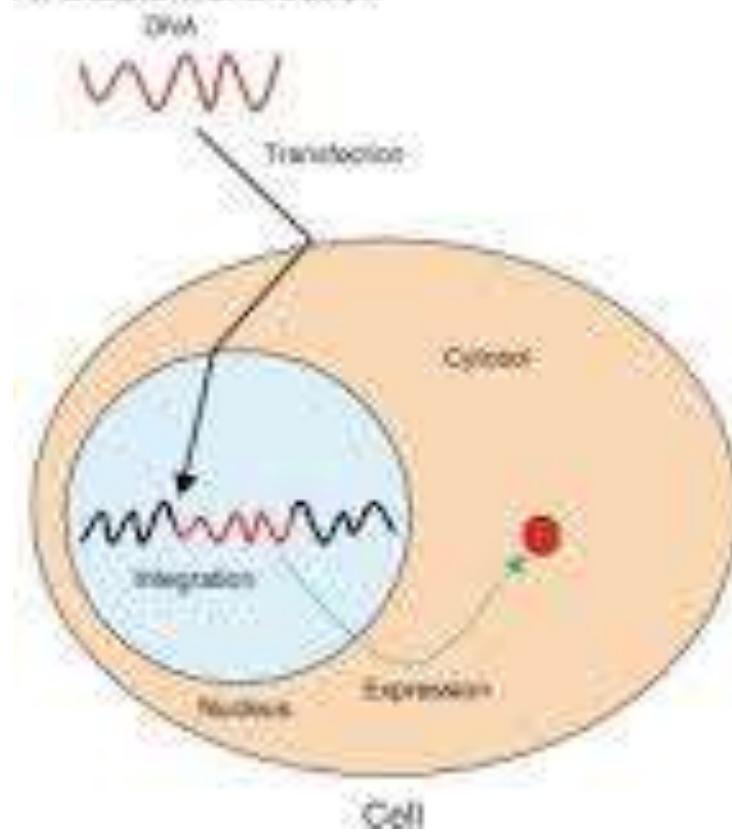
ZNF224 repression activity

La proteina KRAB-zinc finger ZNF224, espressa in cellule eucariotiche, esplica sempre la sua funzione di regolatore negativo trascrizionale (repressore)?

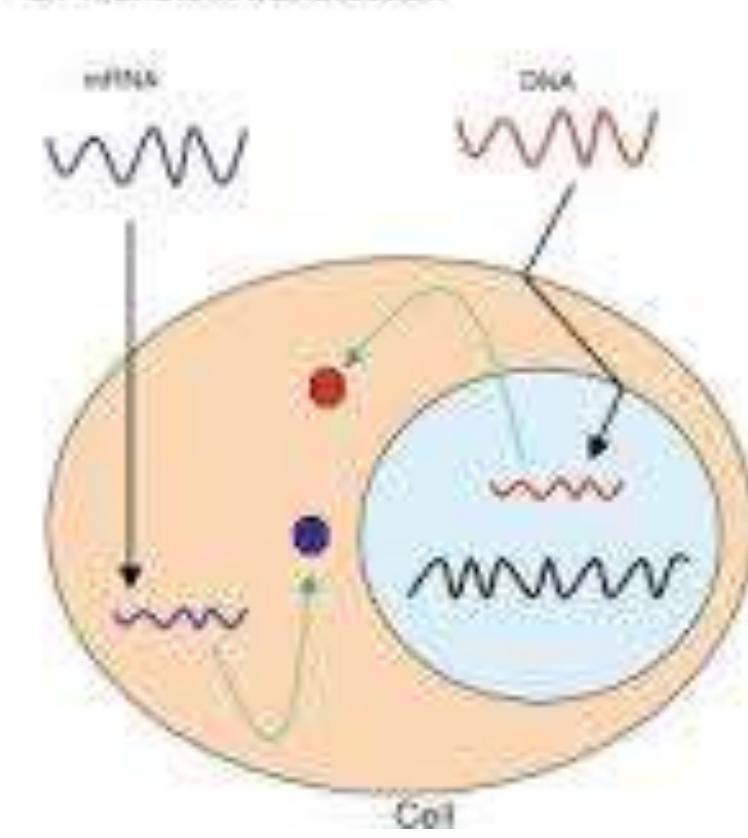
ZNF224 repression activity

Stable or transient ?

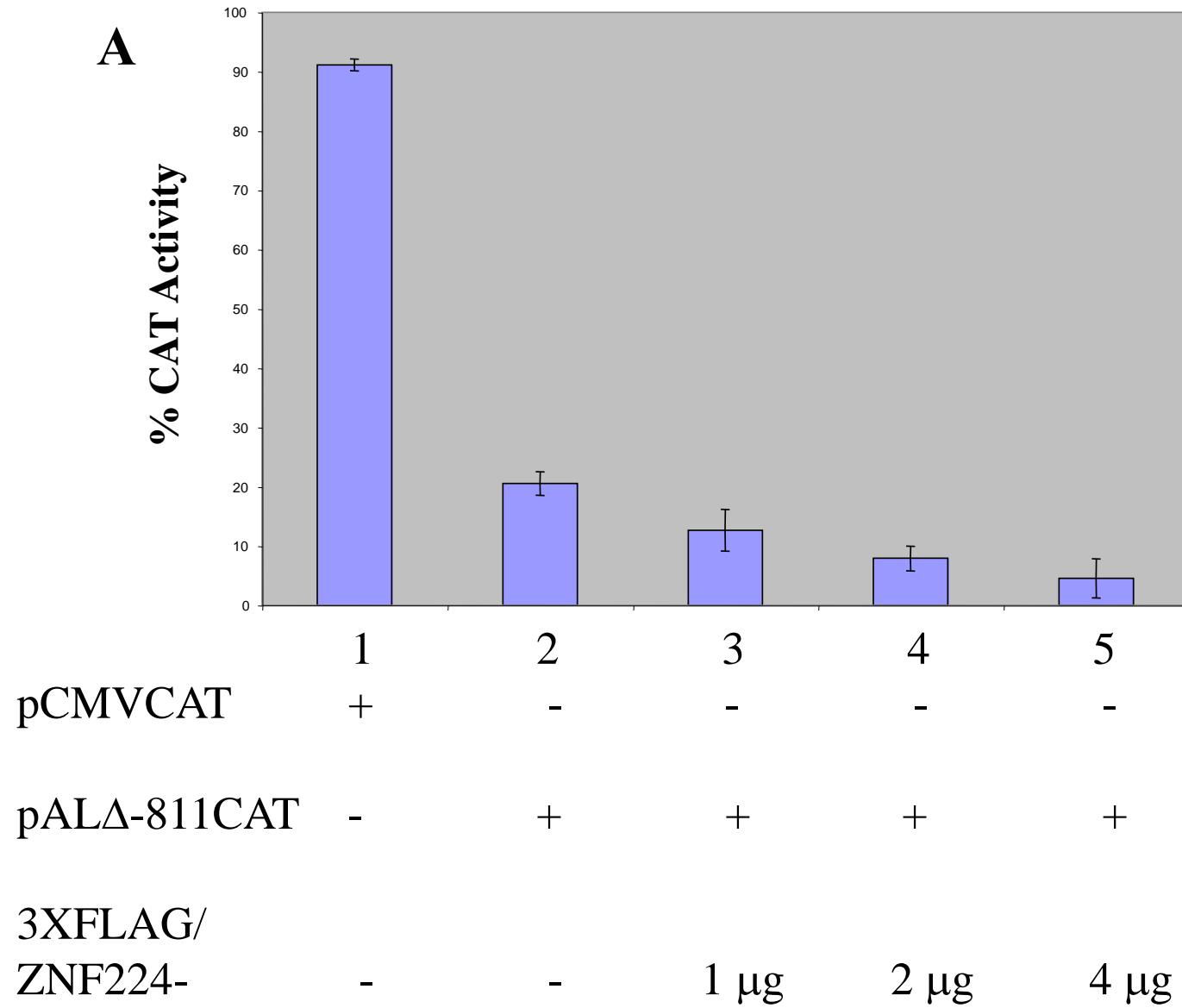
A. Stable transfection



B. Transient transfection

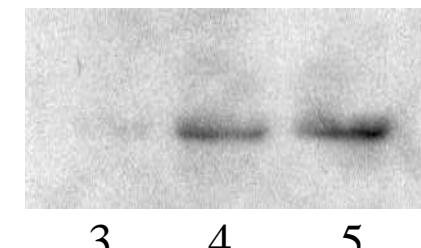


ZNF224 repression activity



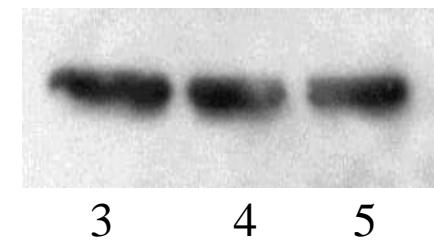
ZNF224 Repressor activity

B



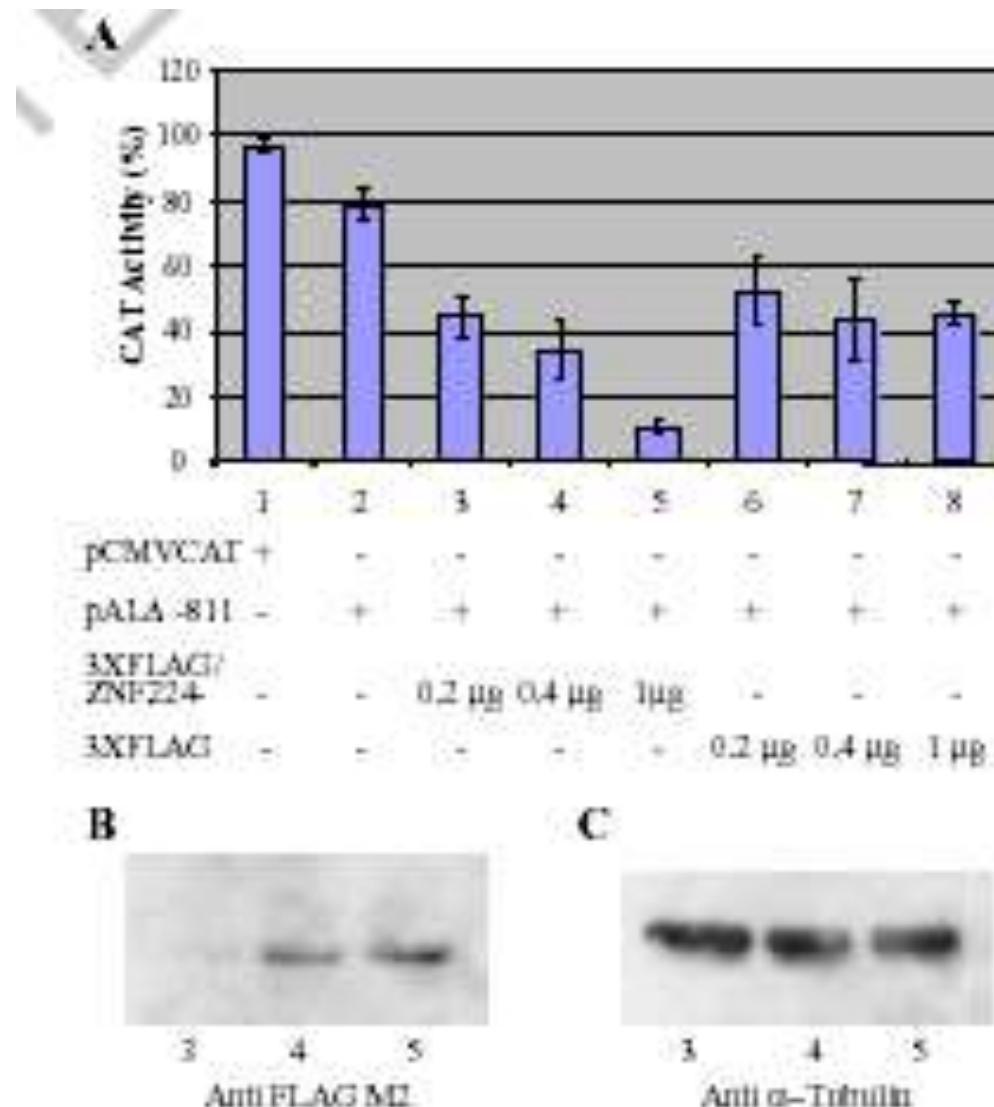
anti-FLAG M2

C



anti- α Tubulin

ZNF224 repression activity



ZNF-224 repression activity

5' HPNRE 2x (reporter gene)

2X NRE



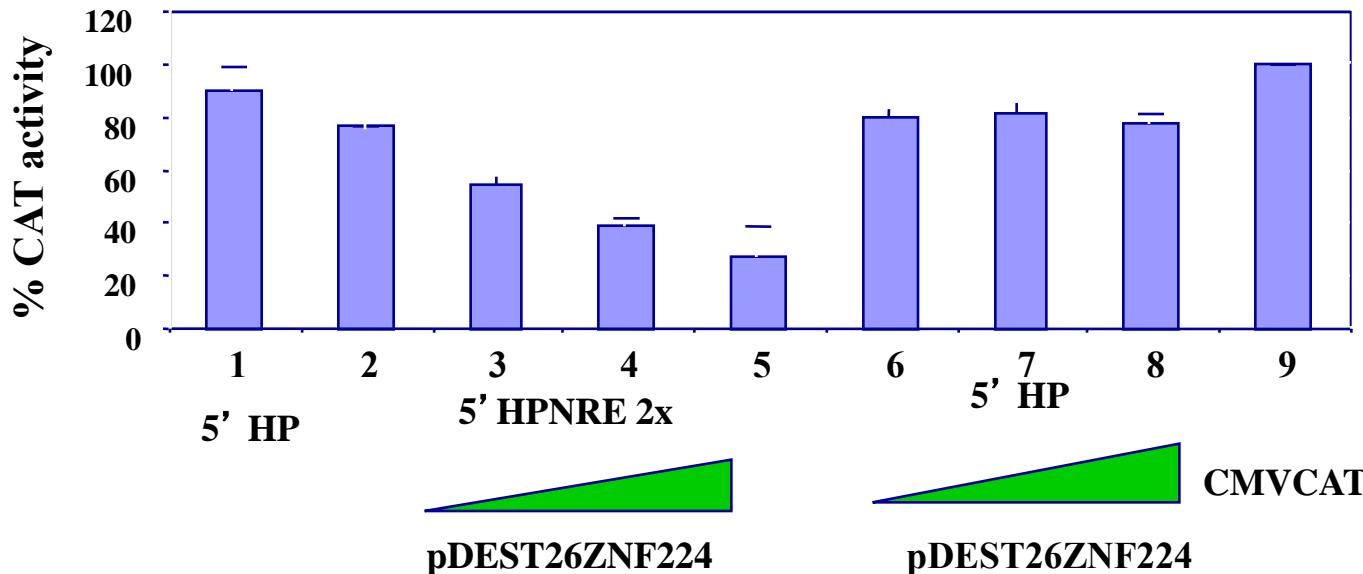
pDEST26 (Mammalian Gateway vector)

CMV promoter

Met

His6

cDNA ZNF224

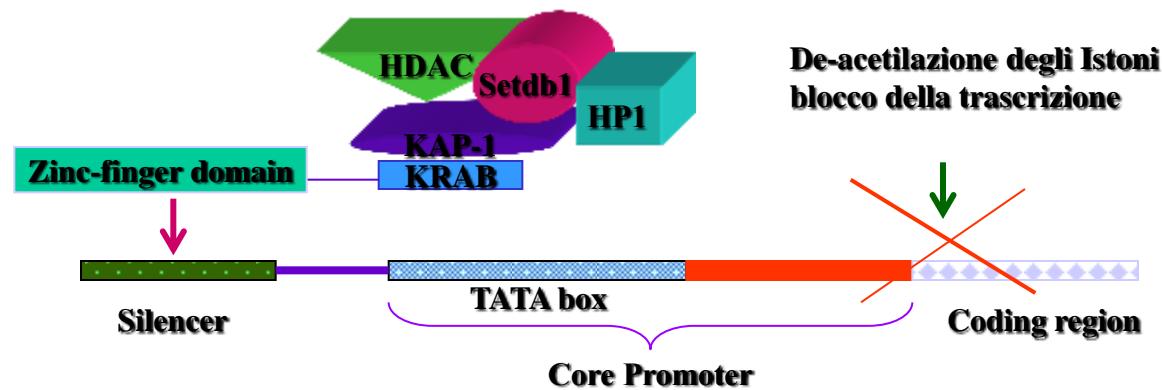


ZNF224 repression activity

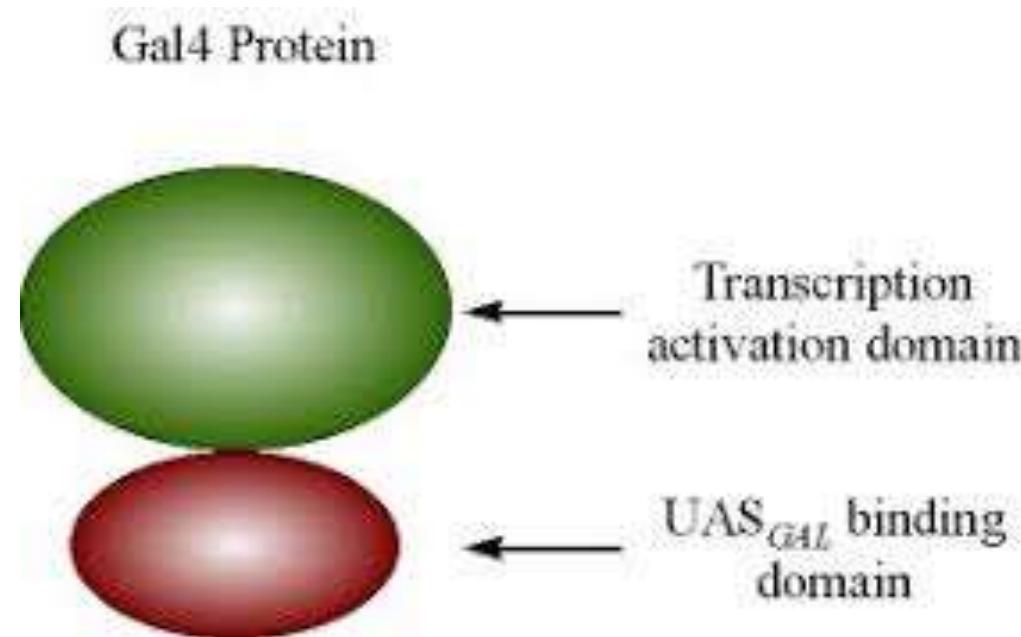
La proteina KRAB-zinc finger ZNF224, espressa in cellule eucariotiche, esplica sempre la sua funzione di regolatore negativo trascrizionale in maniera dominante !

E' forse l'interazione tra la proteina KRAB-zinc finger ZNF224 e il suo co-repressore che risulta cruciale nell' indirizzare l'attività trascrizionale e modularla negativamente ?

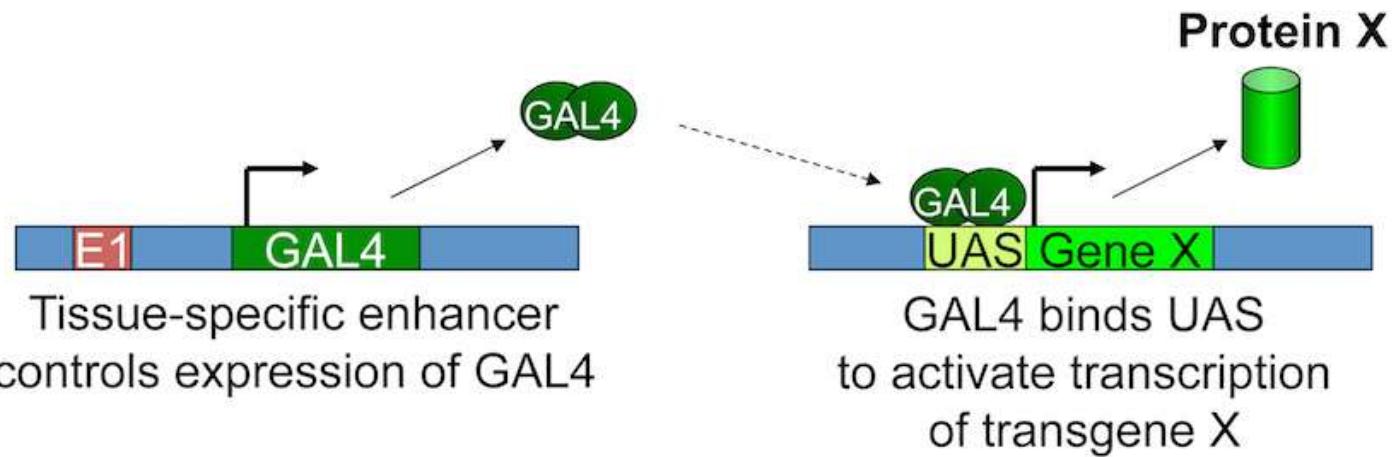
Transcriptional repression is mediated by KRAB A domain



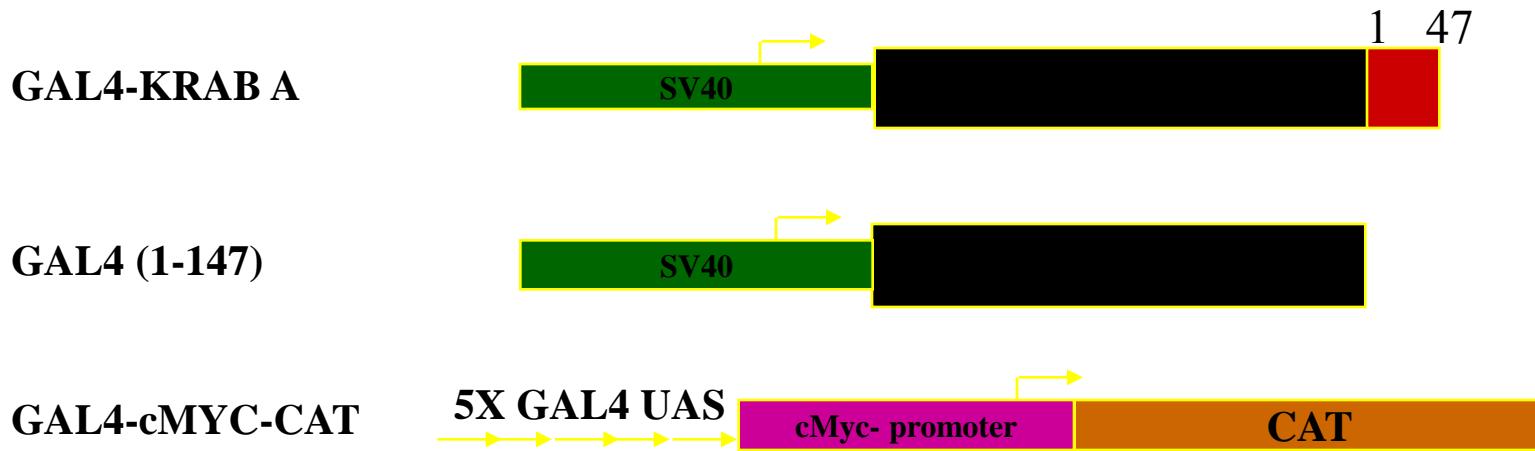
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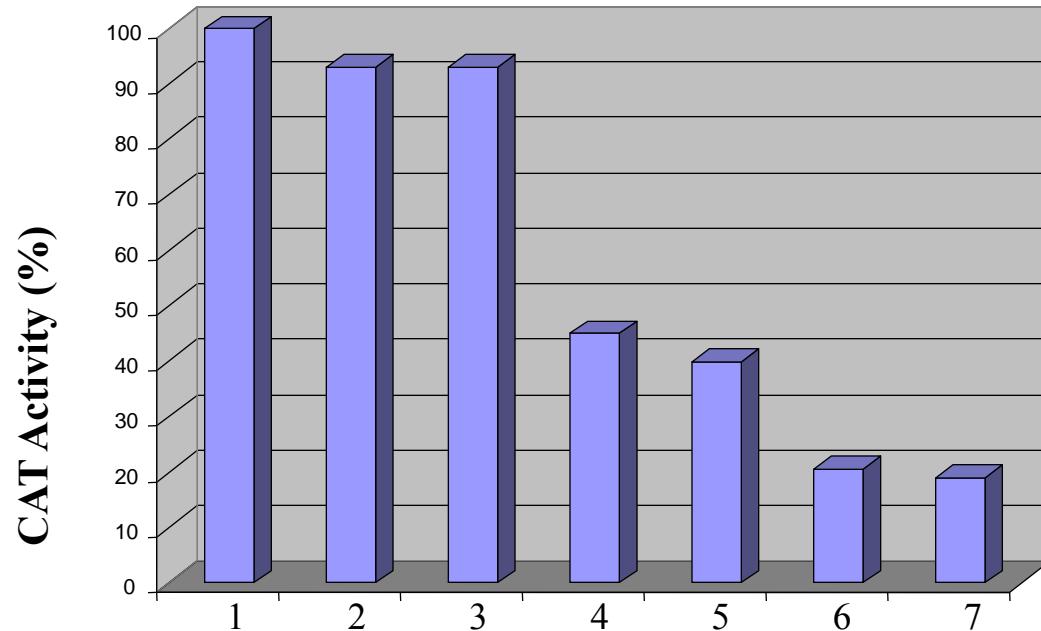
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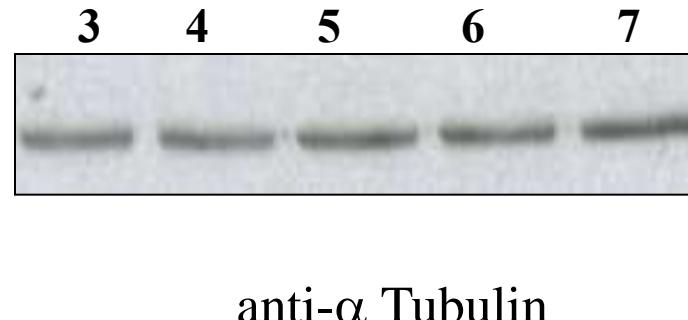
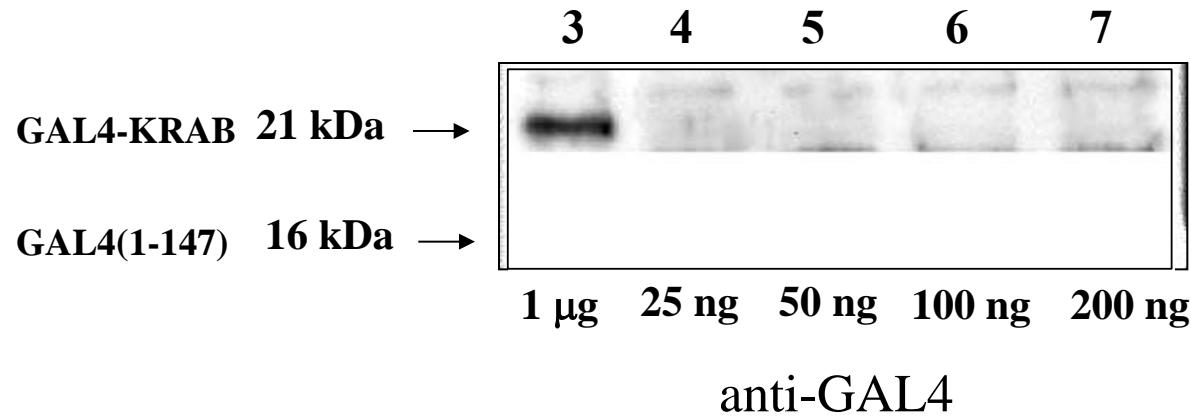


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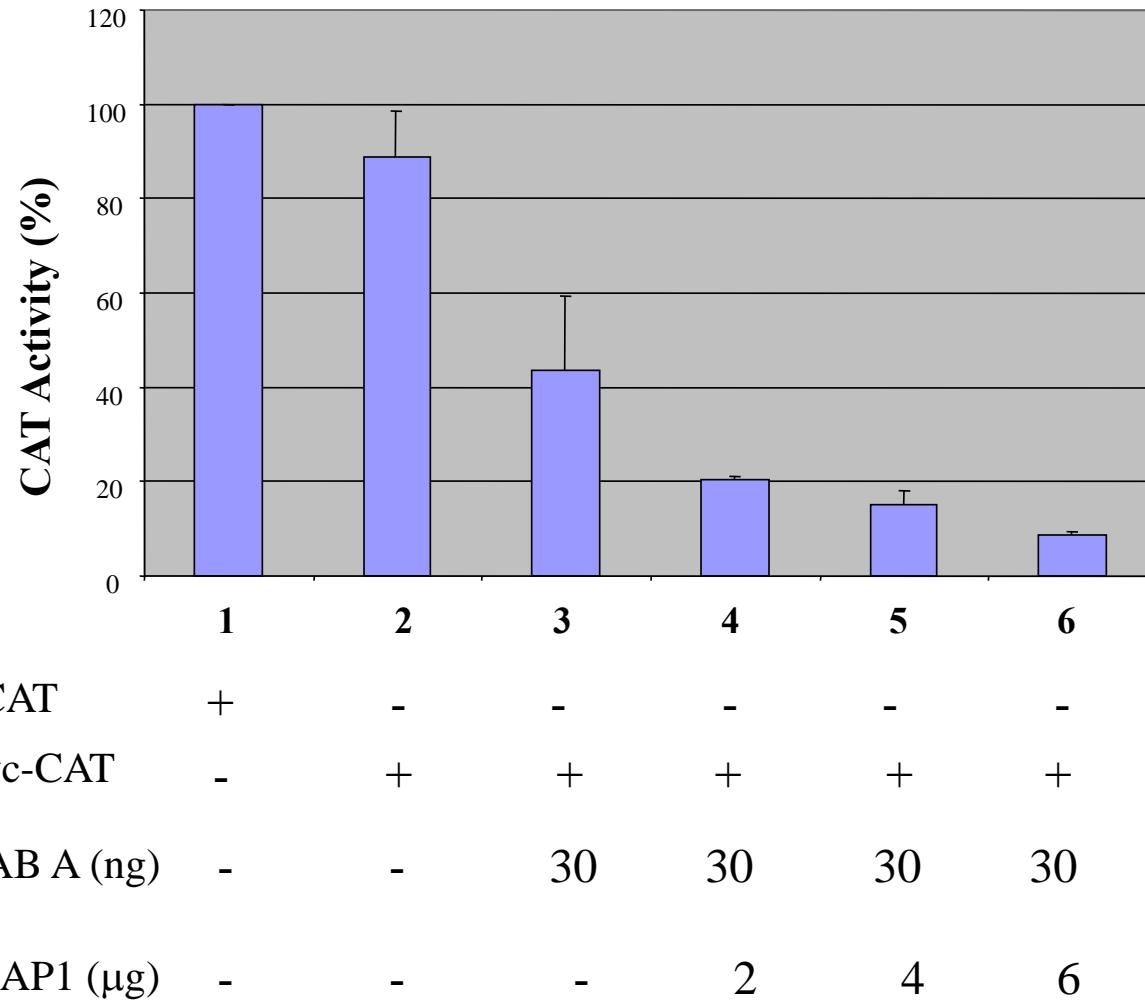


CMV-CAT	+	-	-	-	-	-	-
GAL4-cMYC-CAT	-	+	+	+	+	+	+
GAL4 (1-147) (μ g)	-	-	1	-	-	-	-
GAL4-KRAB A (ng)	-	-	-	25	50	100	200

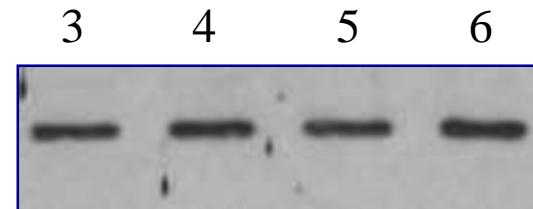
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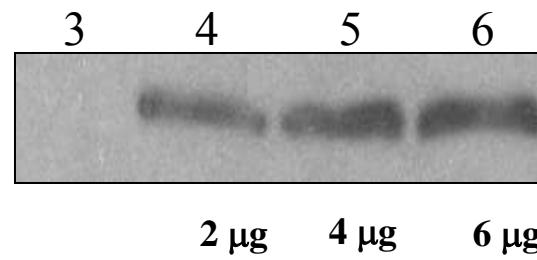
Transcriptional repression is mediated by KRAB A-ZNF224 / KAP1 interaction



Transcriptional repression is mediated by KRAB A-ZNF224 / KAP1 interaction

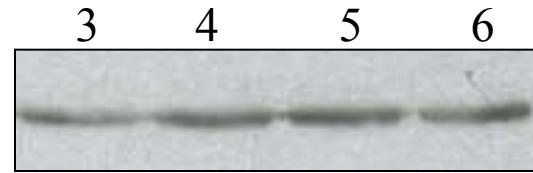


anti-GAL4



2 μg 4 μg 6 μg

anti-FLAG



anti- α Tubulin

Conclusions

- 1) Repression activity of ZNF224 is directed to the homologous as well as the heterologous promoter ;
- 2) The transcriptional repression of ZNF224 requires the KRAB A domain;
- 3) The interaction between the KRAB A domain of ZNF224 and the KAP1 corepressor is the crucial event in the repression;
- 4) The recruitment of the repression complex including KAP1 is necessary for the full ZNF224-mediated repression.

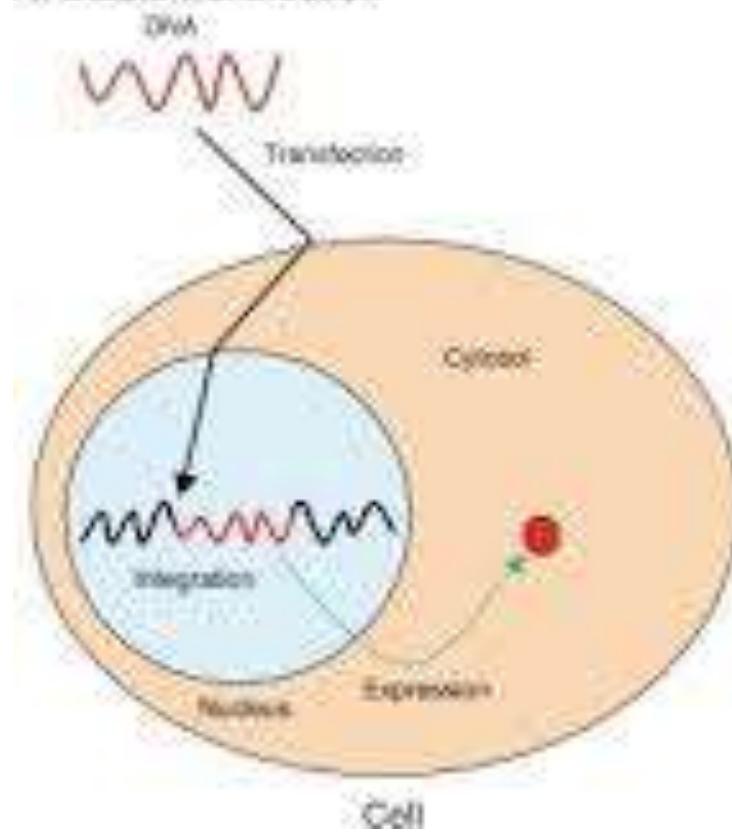
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- transfezione transiente
- transfezione stabile
- espressione costitutiva
- espressione inducibile

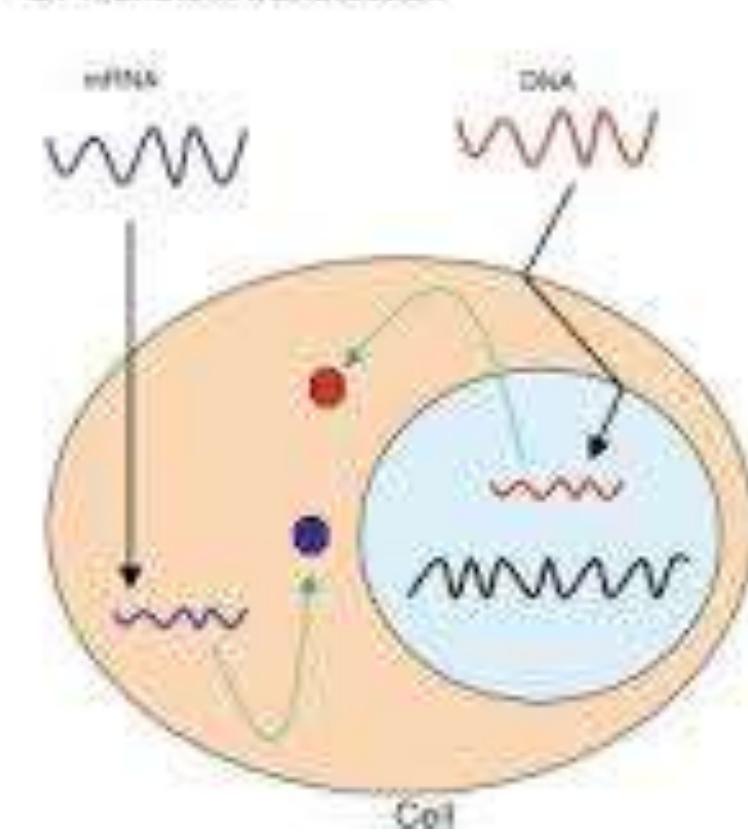
ZNF224 repression activity

Stable or transient ?

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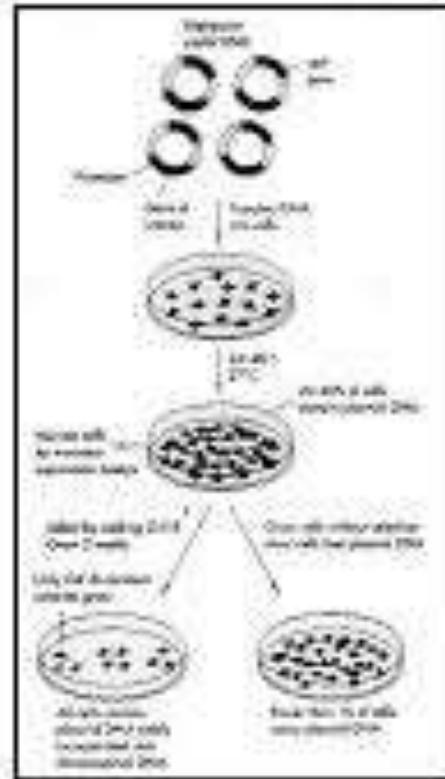


Sistemi d'espressione mediante small-scale transfection in mammalian cells

Transient transfection	Stable transfection
<ul style="list-style-type: none">• Chemical- or electroporation-based• Short-term expression• Manipulation of specific gene/miRNA activity in cell culture• No genomic integration	<ul style="list-style-type: none">• Viral- or micro-injection-based• Sustained expression for long periods of time• Useful where persistent gain-of-function or loss-of-function is required (e.g., xenograft models of tumor growth) and for hard-to-transfect primary cells, or when you need to track and select individual cells• Carries the risk of non-specific integration

Sistemi d'espressione mediante small-scale transfection in mammalian cells

Transfezione stabile



Durante le prime 48 ore dopo la transfezione, fino al 50% delle cellule contengono il DNA esogeno.

In seguito, a causa della degradazione e della diluizione, le cellule che non hanno integrato tale DNA nel loro genoma lo perdono. Per isolare le cellule transfette stabilmente occorre applicare una pressione selettiva, utilizzando un apposito *marker* di selezione.

Sistemi d'espressione mediante small-scale transfection in mammalian cells

Un'altra questione cruciale è :

“ Espressione costitutiva o inducibile ? “

Sistemi d'espressione mediante small-scale transfection in mammalian cells

Regolazione genica nei procarioti

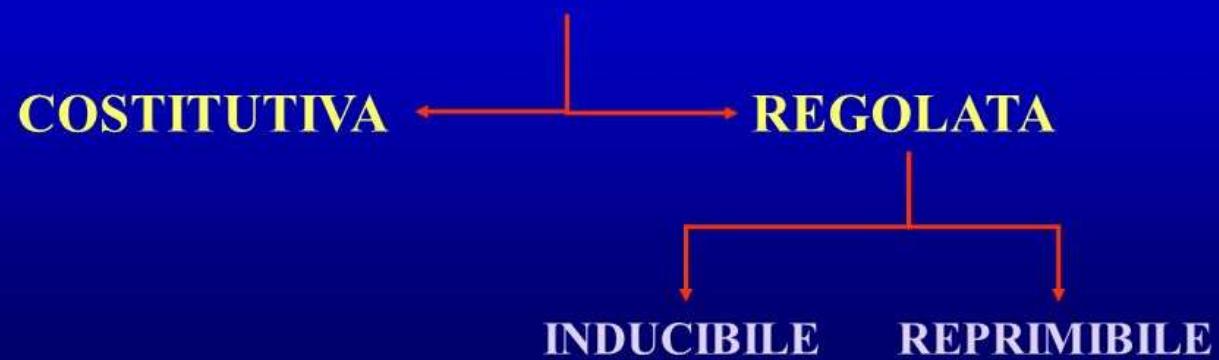
- **Geni costitutivi:** sono costantemente attivi (es. geni che codificano per gli enzimi della glicolisi)
- **Geni regolati:** la loro espressione è regolata in modo tale che la quantità del corrispondente prodotto (proteina o RNA) è controllata in relazione al fabbisogno cellulare (es. sintesi adattativa di enzimi)

Sistemi d'espressione mediante small-scale transfection in mammalian cells

IL CONTROLLO DELL'ESPRESSIONE GENICA

NON TUTTI I PRODOTTI GENICI SONO
NECESSARI ALLO STESSO MOMENTO

L'ESPRESSIONE PUO' ESSERE



Sistemi d'espressione mediante small-scale transfection in mammalian cells

Espressione di proteine esogene:

Quanto ?



Proteina tossica
Alto dosaggio della proteina

Quando ?



Studio delle funzioni durante
lo sviluppo e il differenziamento
(gradienti locali e temporali)

Come ?



Sistemi d'espressione stabili e
modulabili dall'esterno in
eucarioti

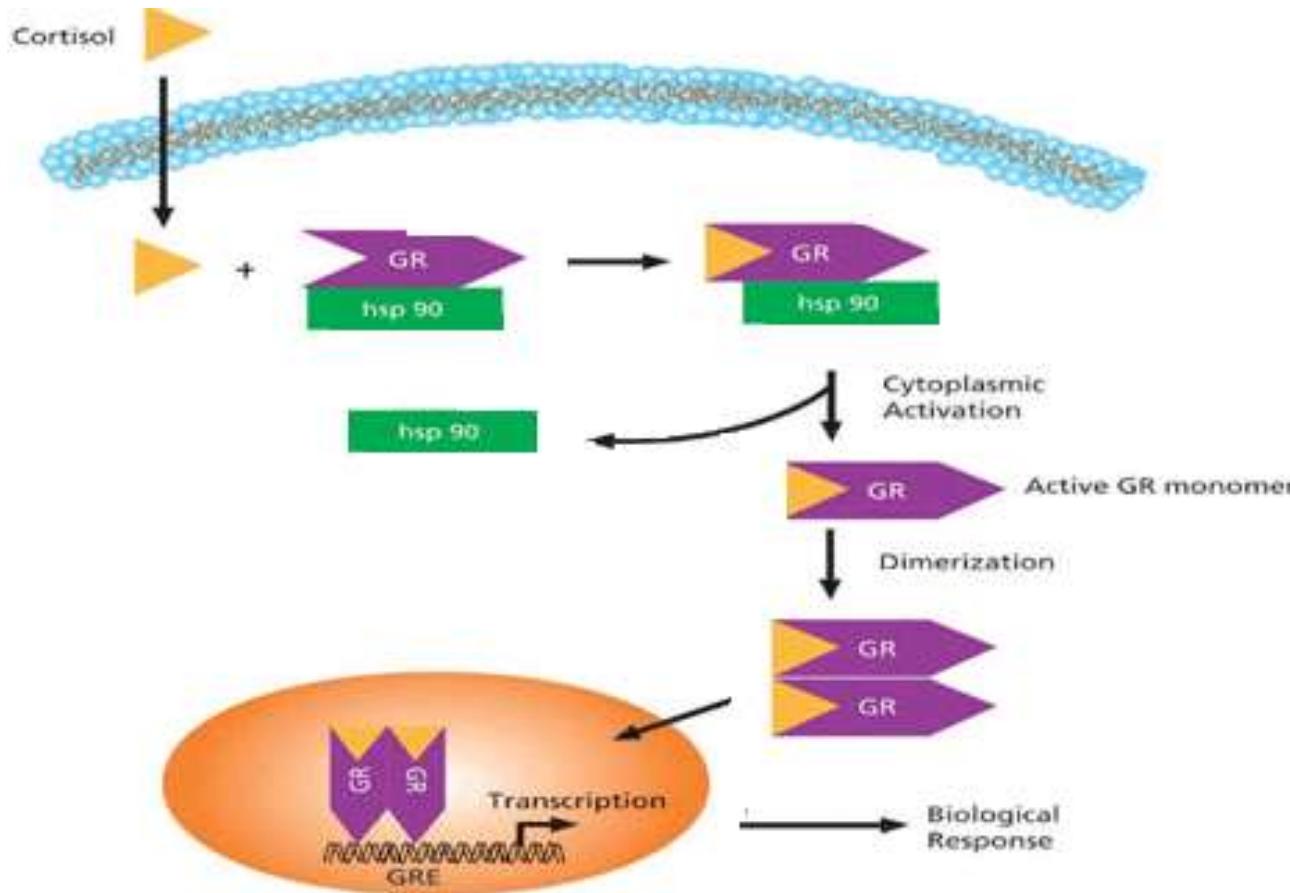
Sistemi d'espressione mediante small-scale transfection in mammalian cells

Espressione inducibile di proteine esogene ha queste caratteristiche :

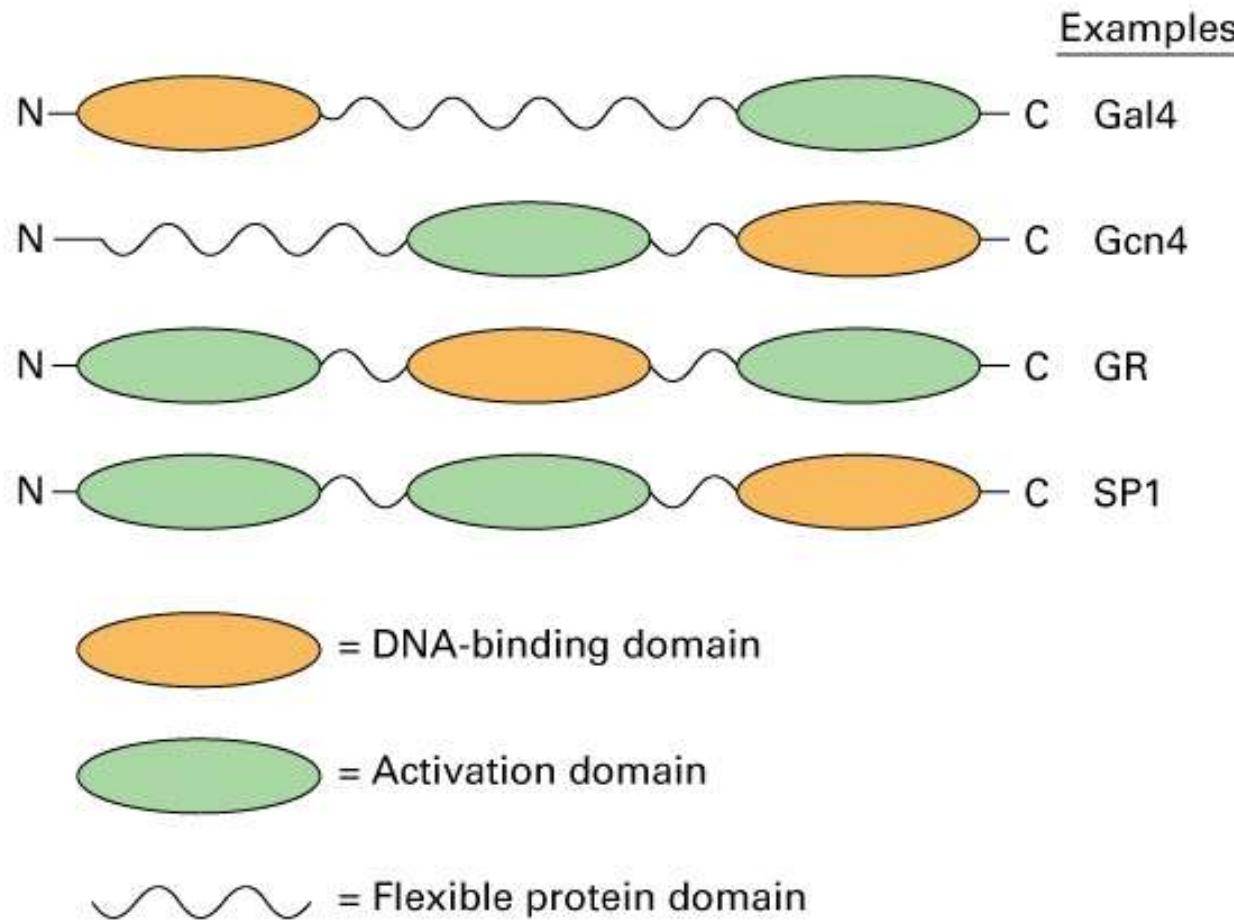
- 1) Specificità : non deve rispondere a segnali endogeni
- 2) Efficienza : deve mostrare bassi livelli in condizioni basali
- 3) Dose-dipendenza : deve essere modulabile



Sistemi d'espressione proteica inducibili



I fattori trascrizionali sono proteine modulari

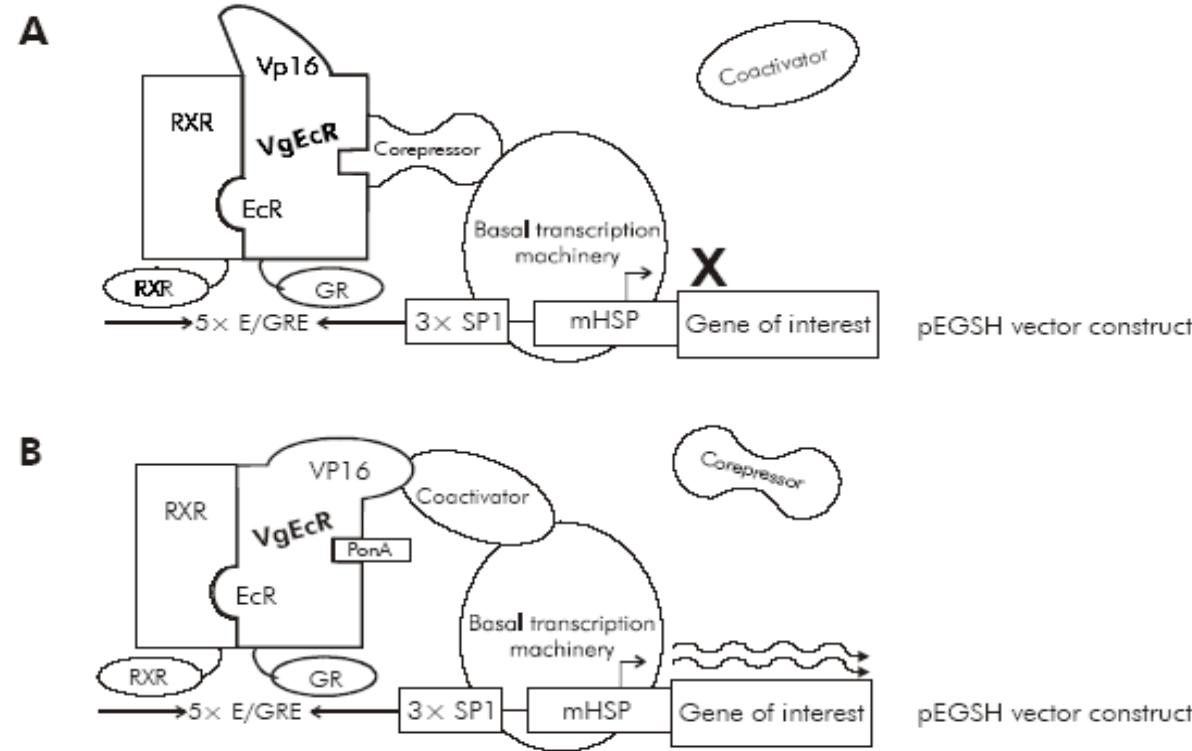




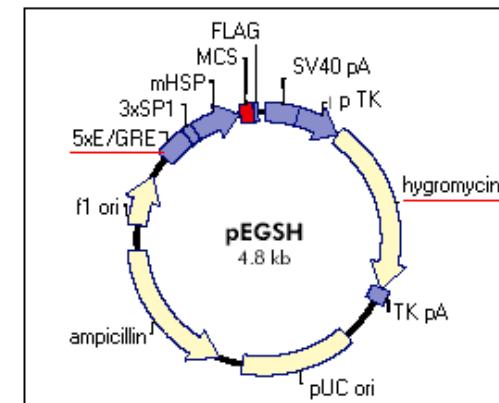
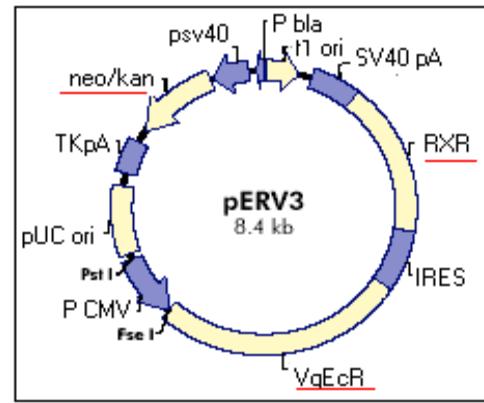
Sistemi d'espressione proteica inducibili

- Il sistema inducibile dall'ecdysone
- Il sistema inducibile del reoswitch
- Il sistema inducibile della rapamicina
- Il sistema inducibile dalla tetraciclina

Il sistema inducibile dall'ecdysone



Il sistema inducibile dall'ecdysone



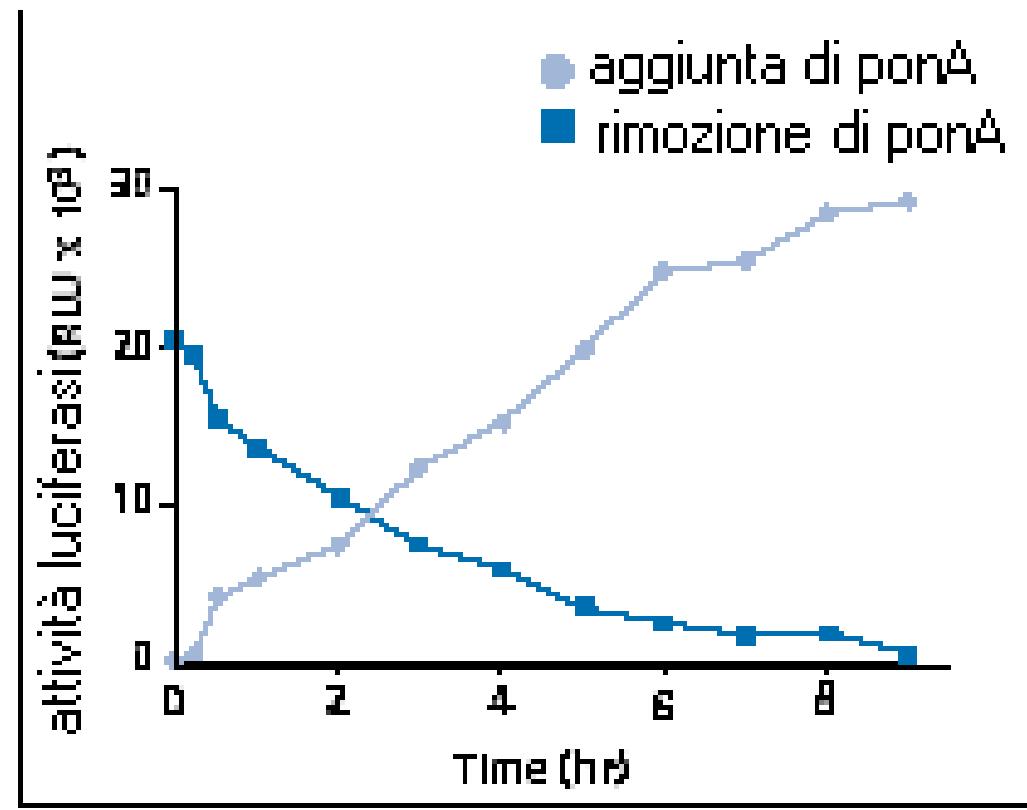
Vettore di espressione

- RXR
- VgEcR
- marker di selezione

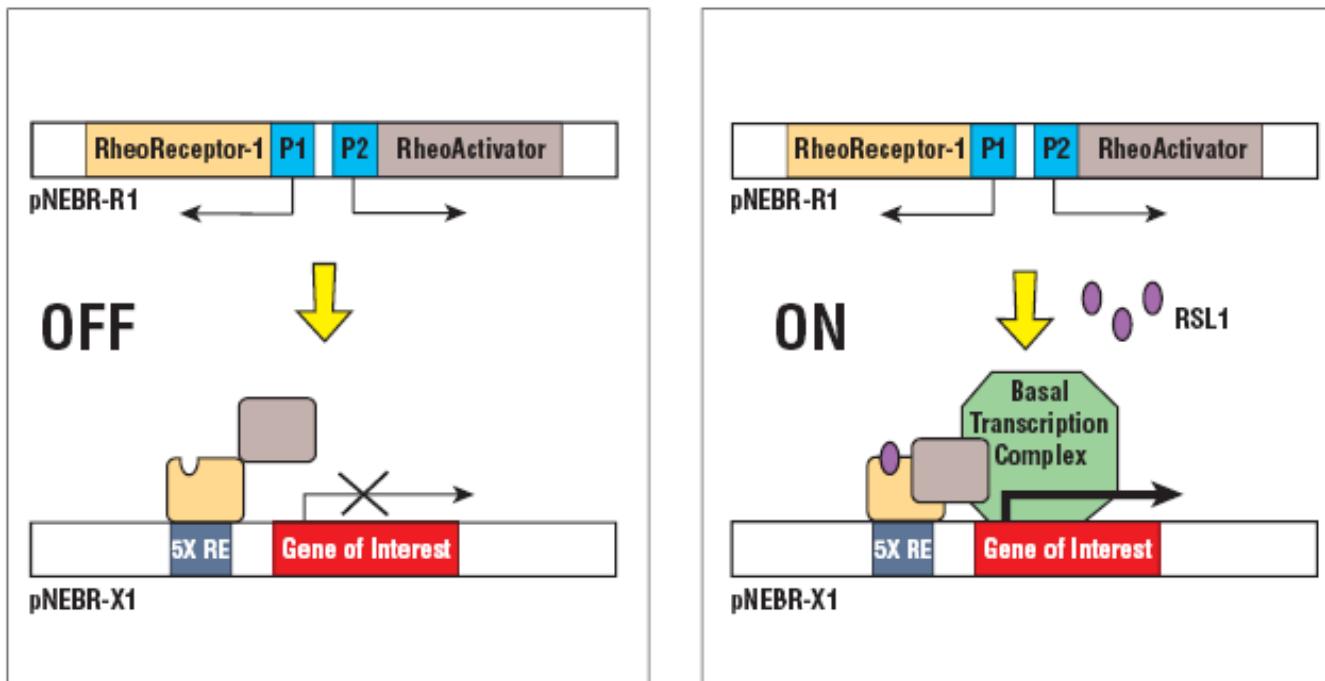
Vettore reporter

- elementi E/GRE
- promotore minimo
- MCS
- gene per la proteina di interesse

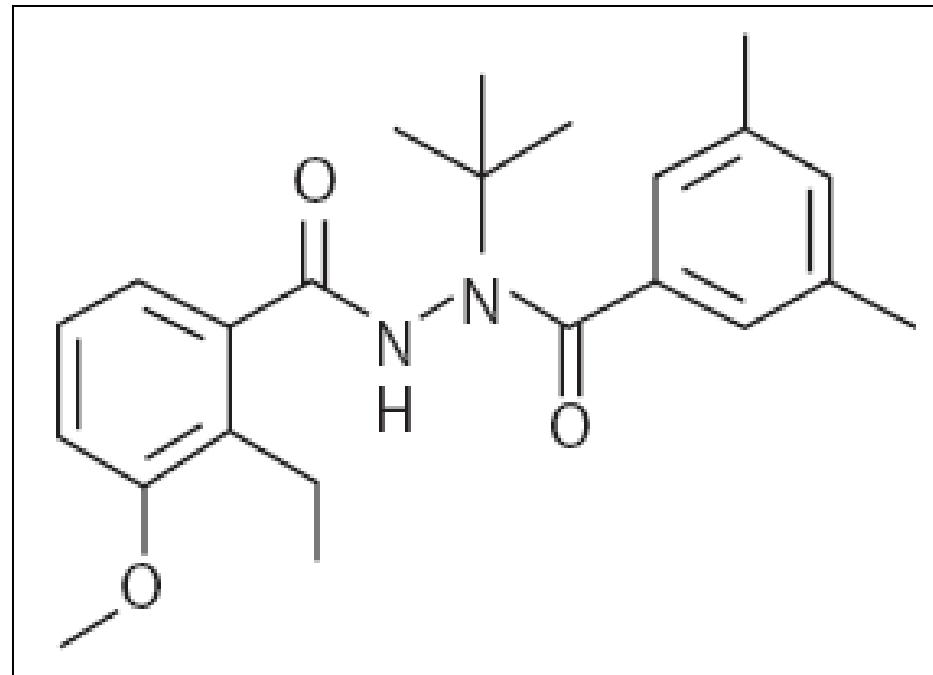
Il sistema inducibile dall'ecdysone



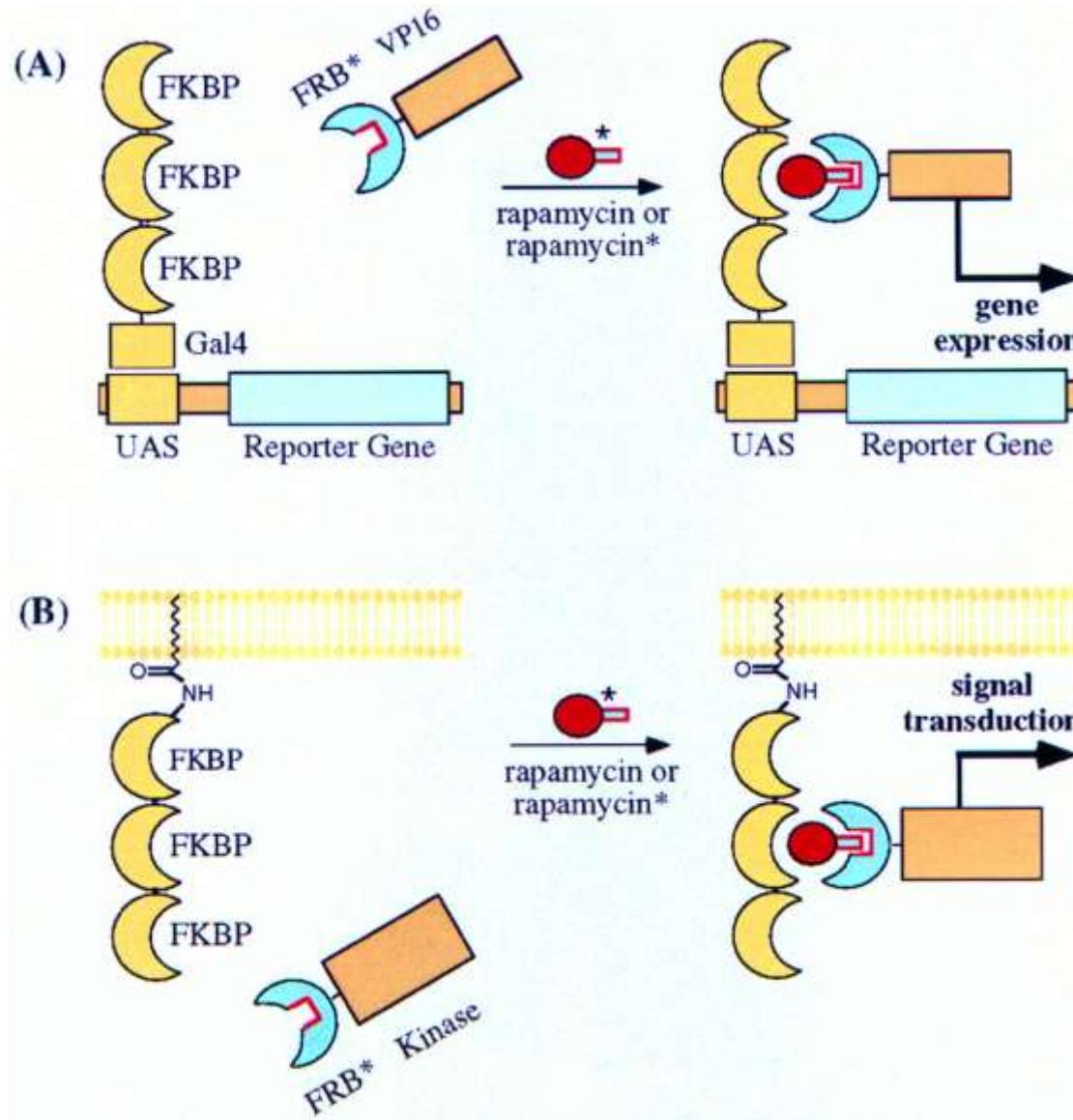
Il sistema inducibile Reoswitch



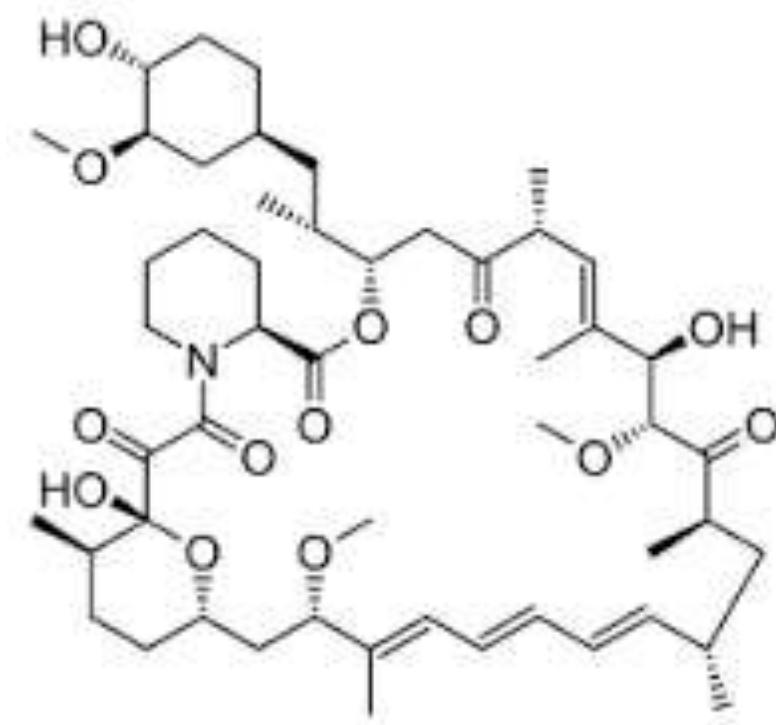
Il sistema inducibile Reoswitch utilizza come induttore N-(2-ethyl-3methoxybenzoyl)-N'-(3,5-dimethylbenzoyl)-N'-tert-butylhydrazine



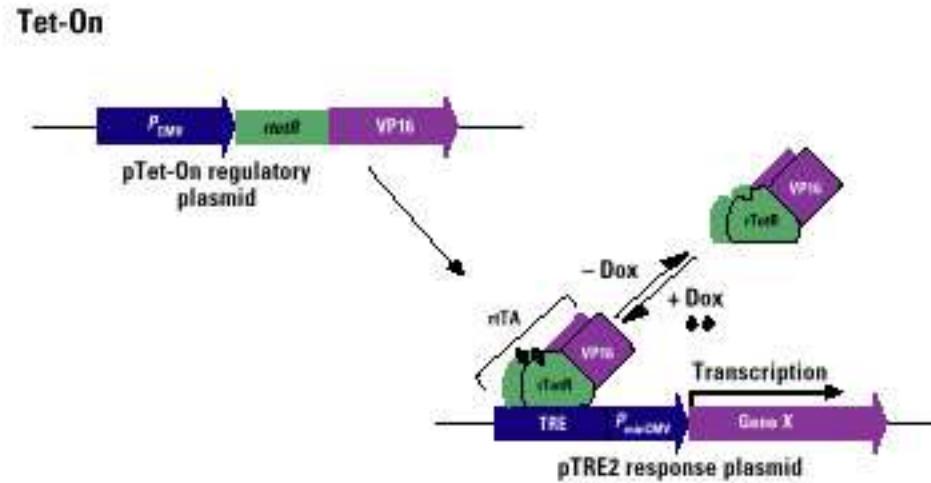
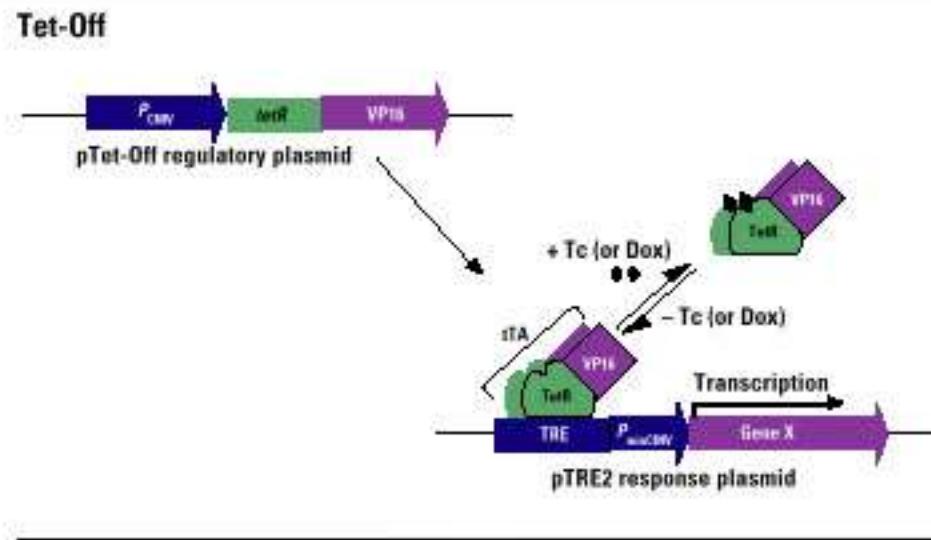
Il sistema inducibile della rapamicina



Il sistema inducibile della rapamicina

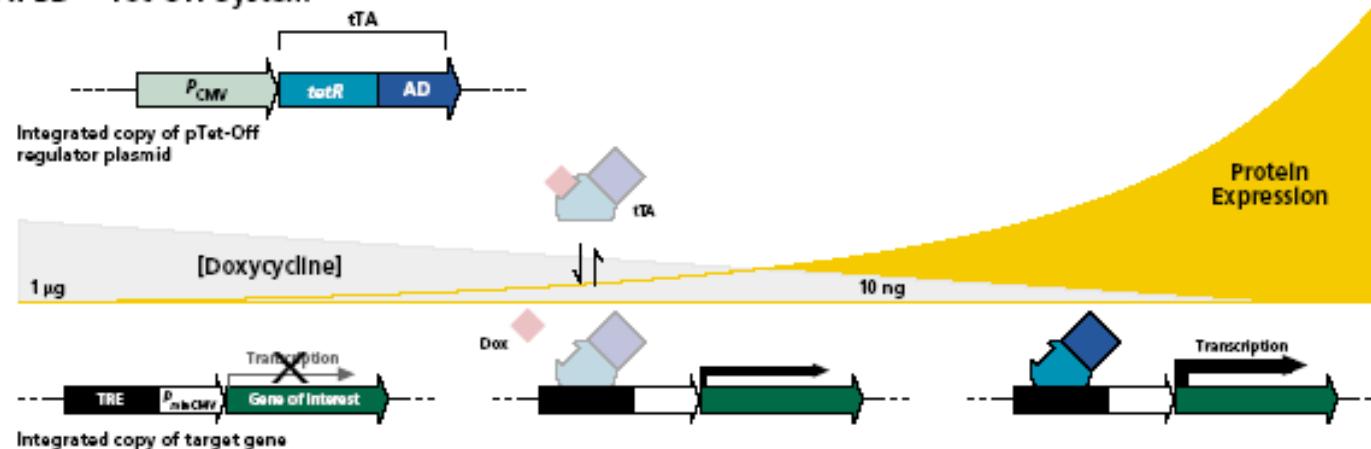


Sistema inducibile TET-ON / TET-OFF

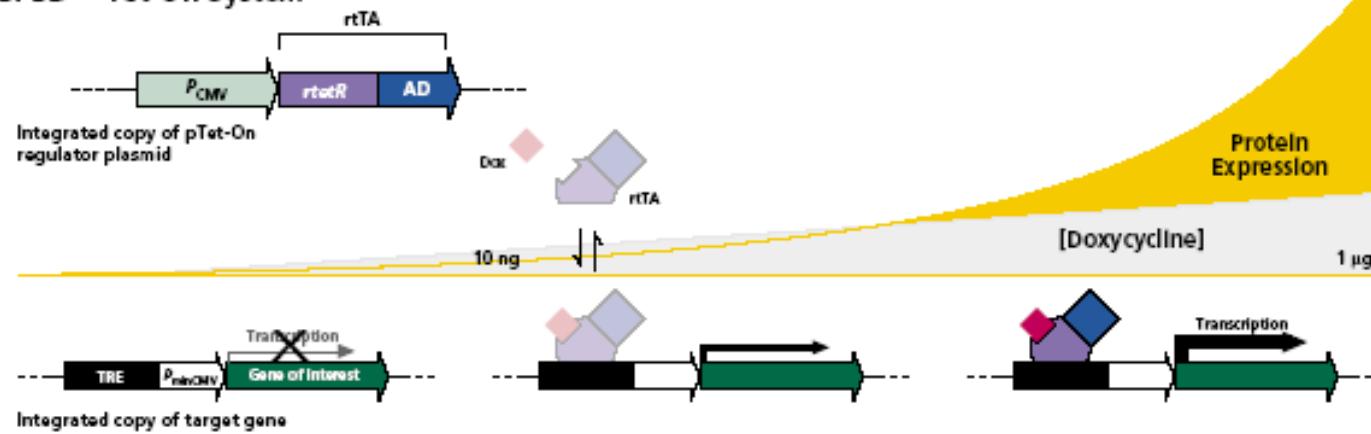


Sistema inducibile TET-ON / TET-OFF

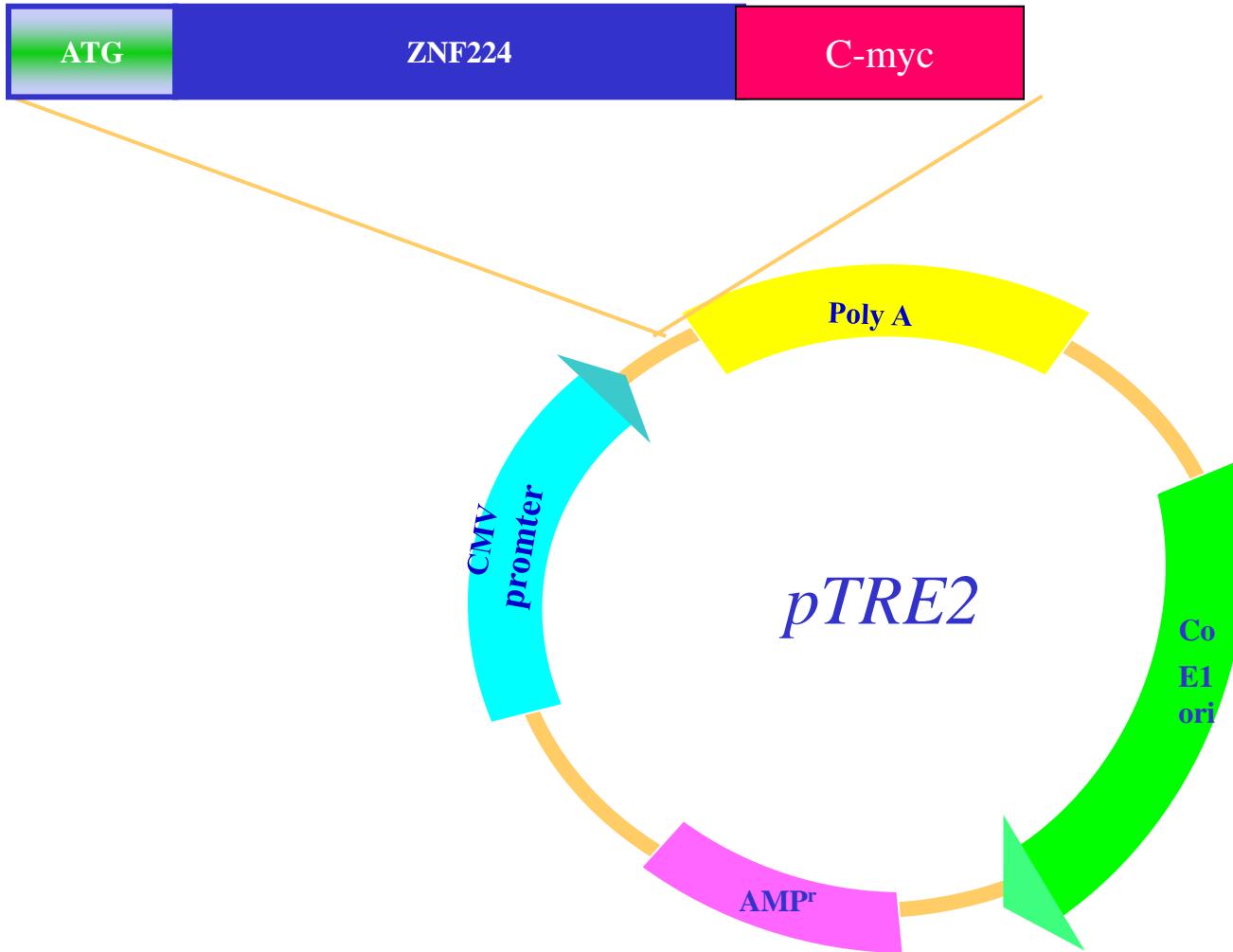
A. BD™ Tet-Off System



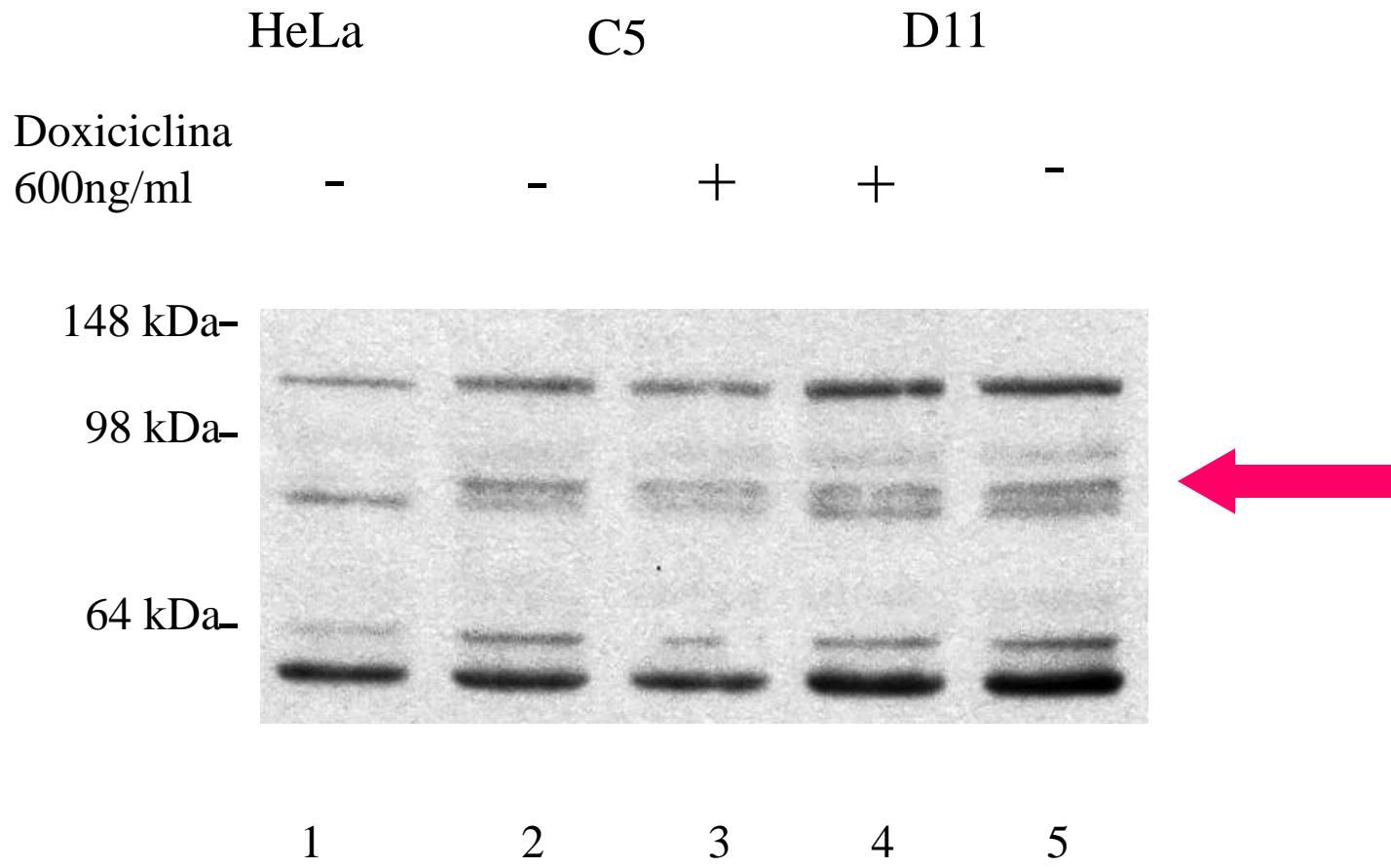
B. BD™ Tet-On System



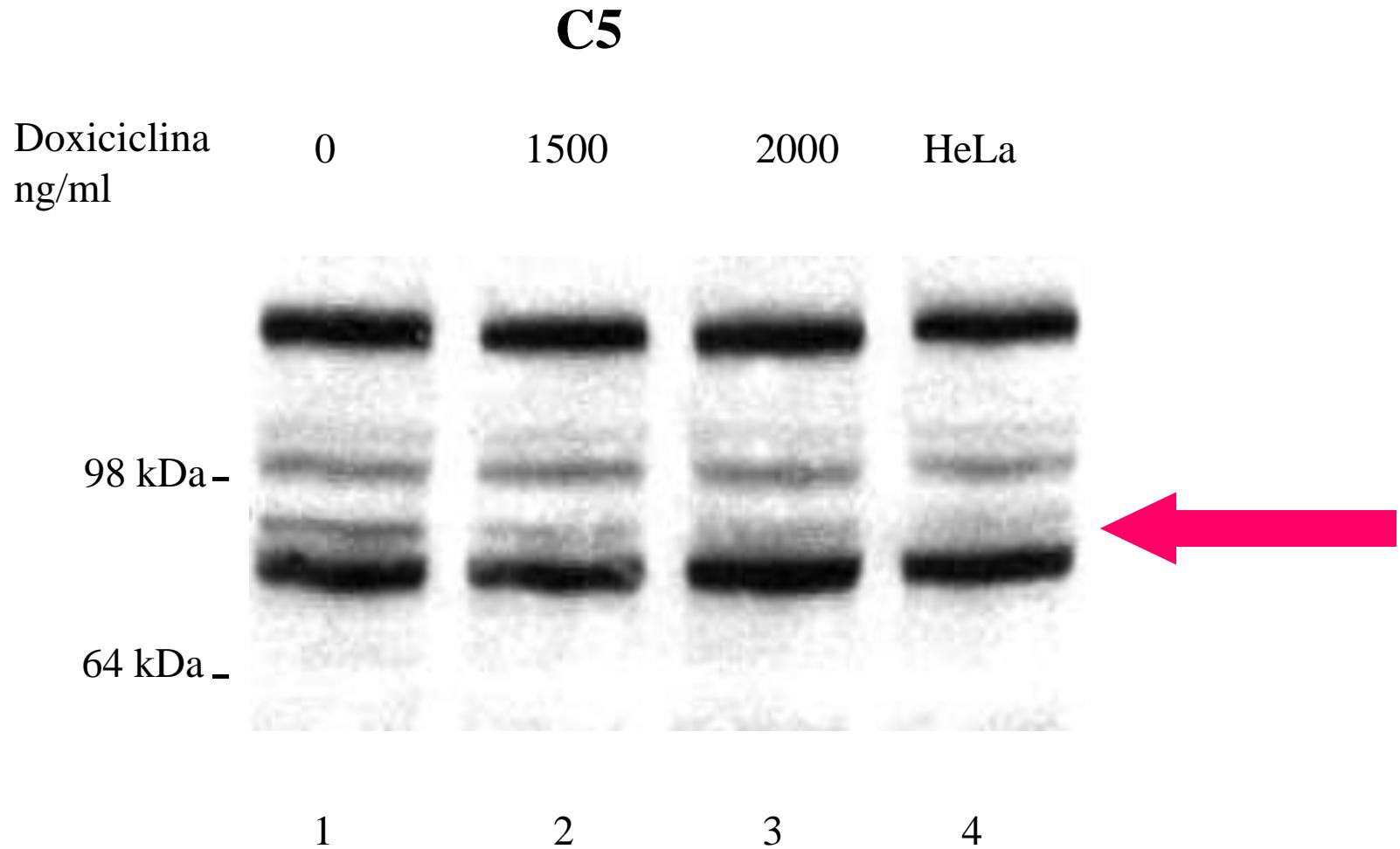
Il cDNA codificante per la proteina di fusione ZNF224-myc è stato clonato nel plasmide pTRE2



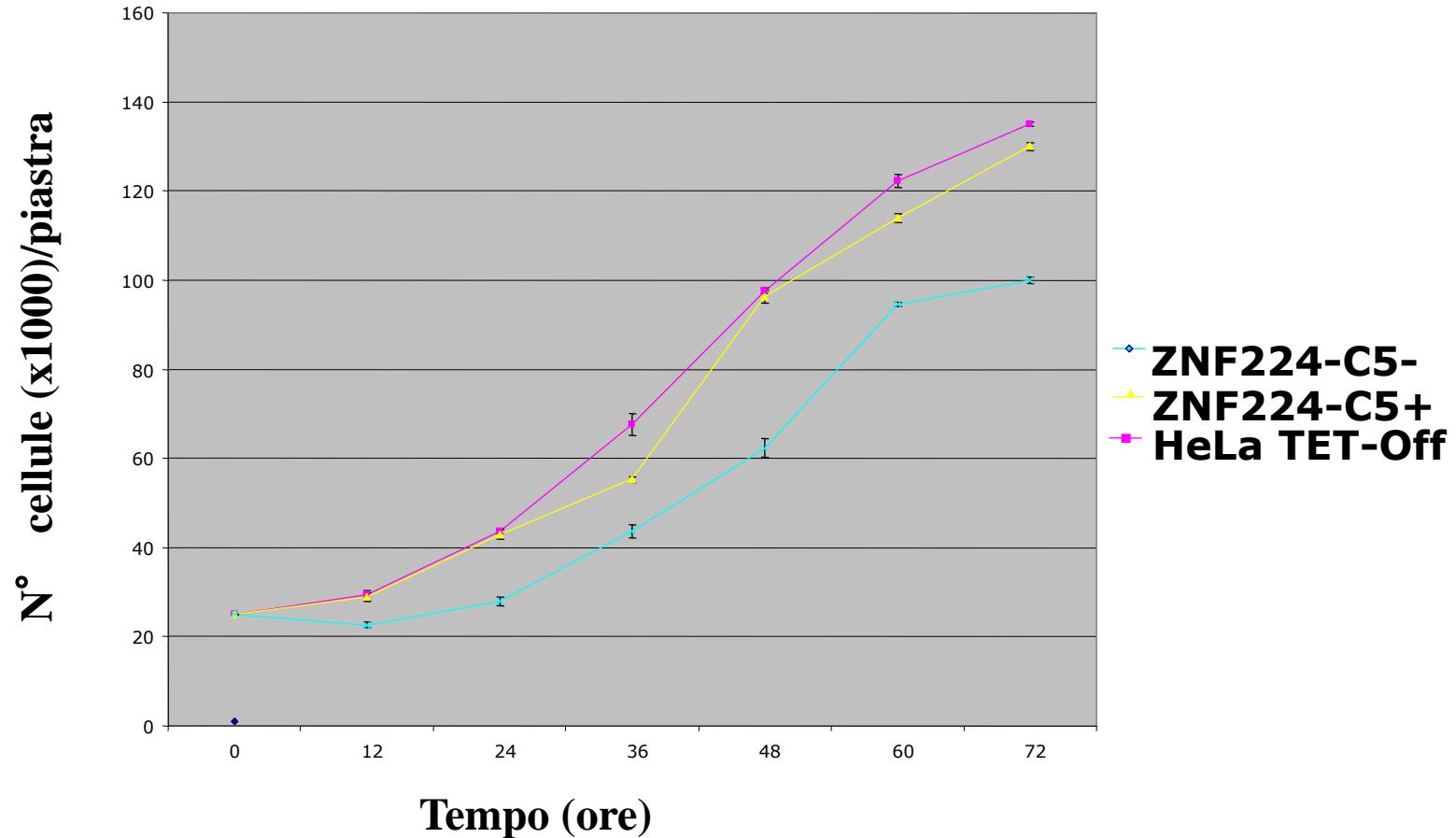
I cloni C5 e D11 overesprimono la proteina di fusione ZNF224-myc



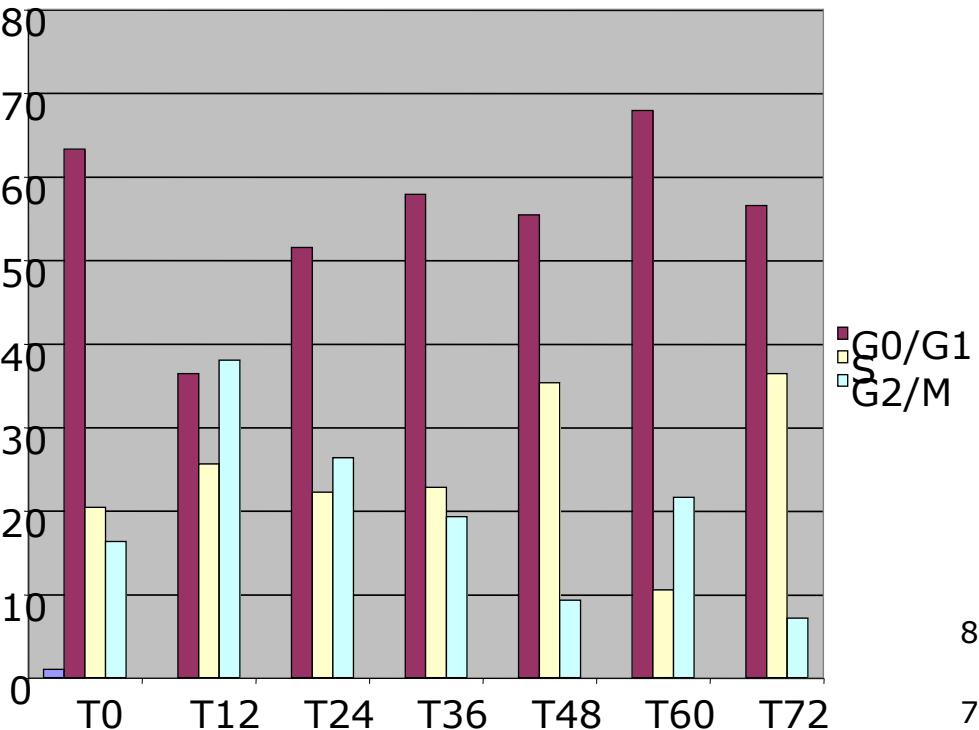
La overespressione di ZNF224-myc viene repressa con concentrazione di doxiciclina di 2000ng/ml



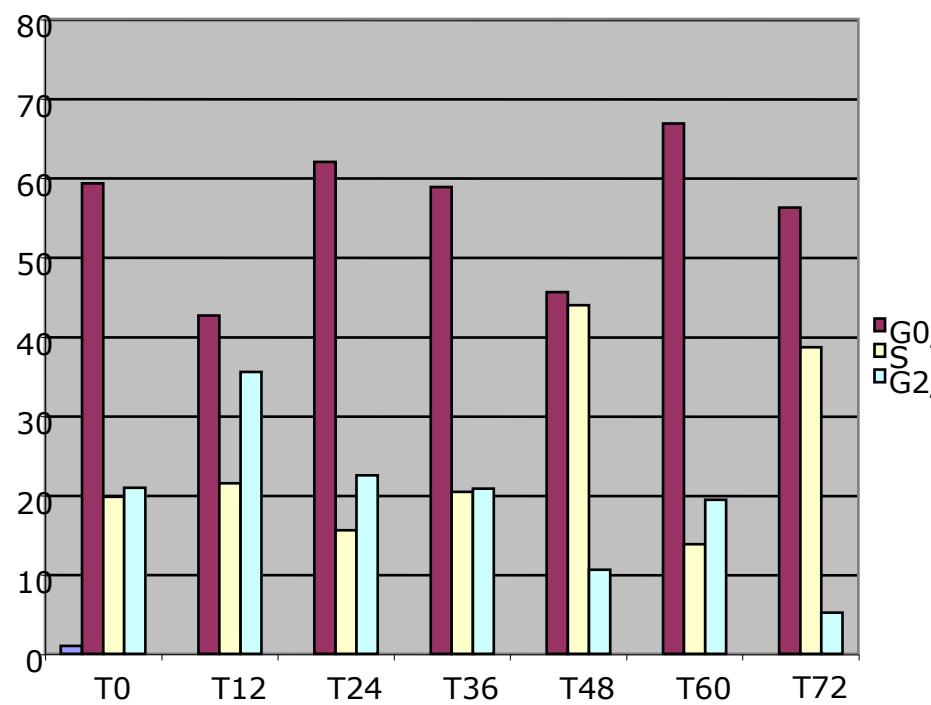
L'overespressione di ZNF224 provoca una diminuzione del numero di cellule C5-



C5-Cytofl.



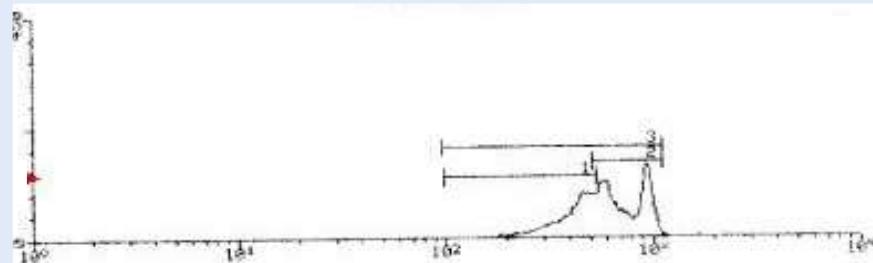
C5+cytofl.



L'overespressione di ZNF224 inibisce la proliferazione cellulare evidenziando un aumento della popolazione apoptotica cellulare subG0/G1

C5- T24

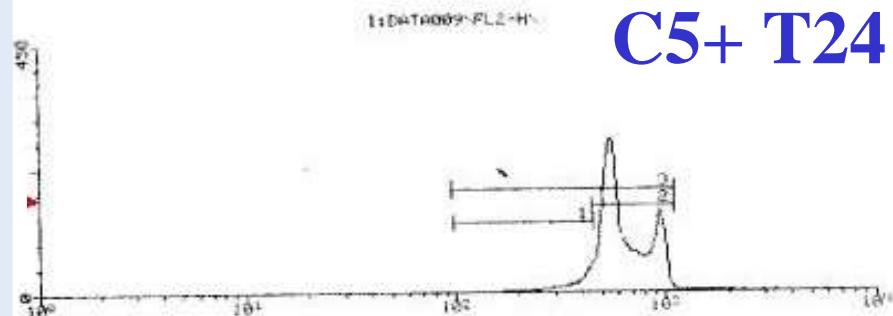
41.06%



1:DATA009-FL2-H			
--- Arithmetic Histogram Statistics			
Parameter	FL2-H	Gate G1= R1	
M	Left, Right	Events	%
0	1.00, 9910	9800	100.00
1	99.10, 533	4824	41.06
2	514, 1114	6200	53.26
3	97.33, 1114	9791	99.99

9.24%

C5+ T24



1:DATA009-FL2-H			
--- Arithmetic Histogram Statistics			
Parameter	FL2-H	Gate G1= R1	
M	Left, Right	Events	%
0	1.00, 9910	12081	100.00
1	98.21, 453	1129	9.34
2	445, 1094	11612	91.15
3	96.46, 1094	11996	99.21

Final Conclusions

- 1) Repression activity of ZNF224 is directed to the homologous as well as the heterologous promoter ;
- 2) The transcriptional repression of ZNF224 requires the KRAB A domain;
- 3) The interaction between the KRAB A domain of ZNF224 and the KAP1 corepressor is the crucial event in the repression;
- 4) The recruitment of the repression complex including KAP1 is necessary for the full ZNF224-mediated repression;
- 5) The overexpression of ZNF224 induces a G0/G1 cell proliferation arrest through a specific increase of subG0/G1 cell subpopulation.