Expresión de la información genética en eucariotas

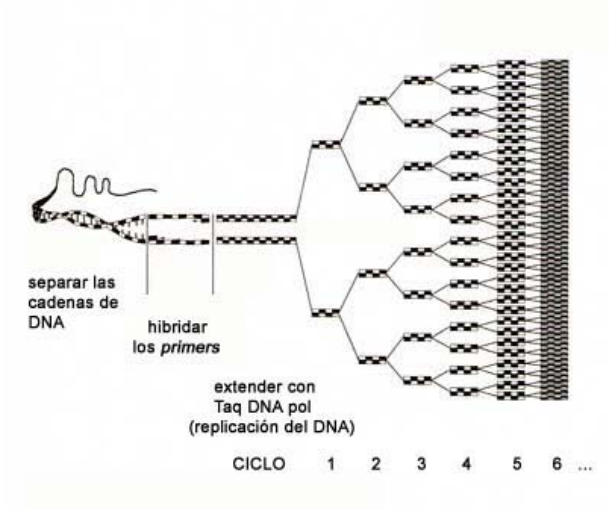
- similitudes y diferencias con procariotas
- metodologías de estudio 2

Víctor Romanowski, 2013

Polymerase chain reaction (PCR)

- The polymerase chain reaction (PCR) can be used to amplify rare specific DNA sequences from a complex mixture when the ends of the sequence are known
- PCR amplification of mutant alleles allows detection of human genetic diseases
- DNA sequences can be amplified by PCR for use in cloning, as probes, and in forensics

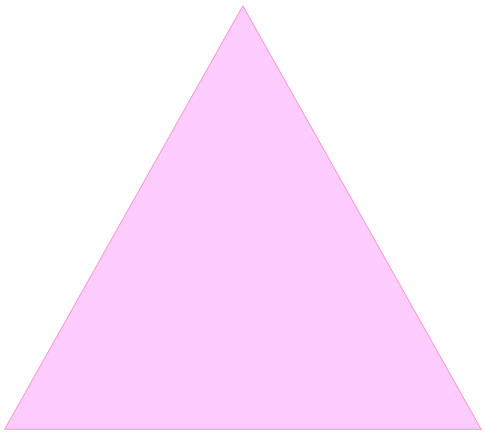
PCR: *Polymerase Chain Reaction*



Ciclos	Copias
1	2
2	4
4	16
10	1.024
15	32.768
20	1.048.576
25	33.554.432
30	1.073.741.824

CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824

Número de copias de la secuencia blanco (dsDNA)



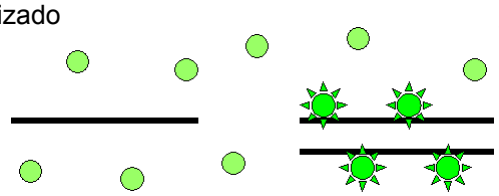
Real time RT-PCR
qRT-PCR

q = quantitative

Cuantificación directa y contínua **durante** la amplificación

Alternativas de uso de fluoróforos:
agentes intercalantes o acoplados a sondas o *primers*

Existe una proporcionalidad directa entre la cantidad moléculas de fluoróforo que emiten luz y la cantidad de moléculas de DNA sintetizado



SYBR green

CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
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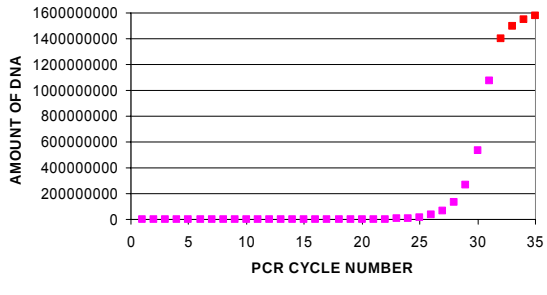
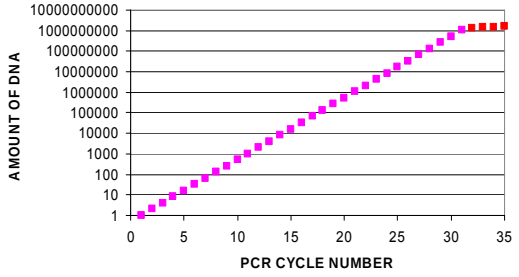
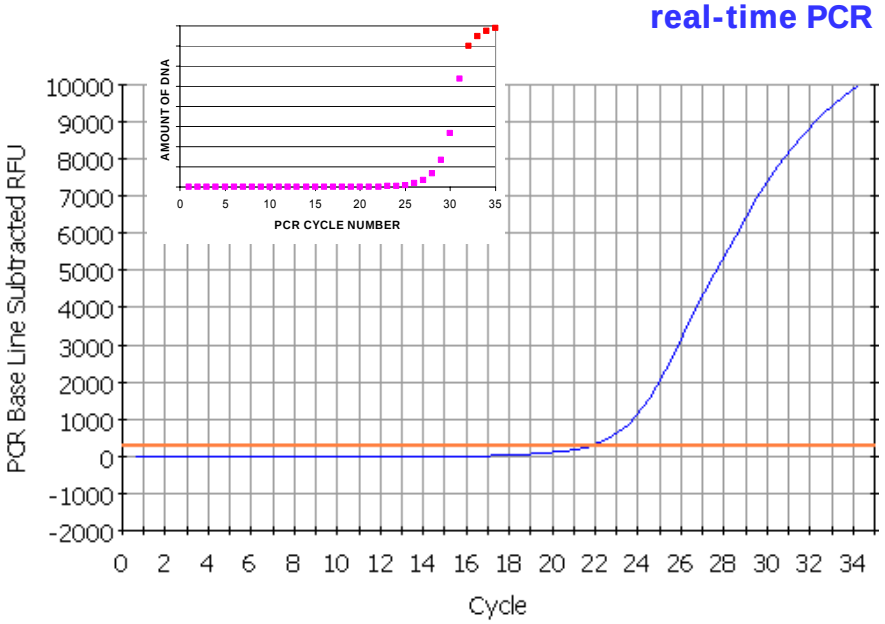
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,550,000,000
34	1,580,000,000





real-time PCR

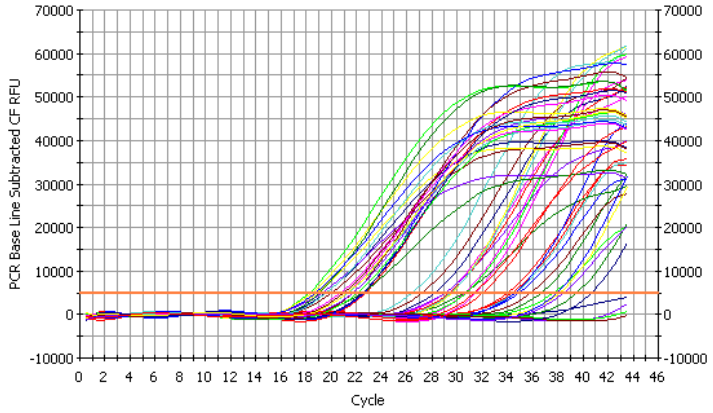


real-time PCR trace for a single well on a 96-well plate, cycles are shown along the X-axis, and arbitrary fluorescence units (actually these are fold increase over background fluorescence) are shown on the Y-axis

PCR en tiempo real (Real time PCR o qPCR)

RNA ⟶ cDNA ⟶ PCR con cebadores específicos en presencia de un compuesto fluorescente

iCycler



BIO-RAD

Técnicas para identificar interacciones DNA-proteína

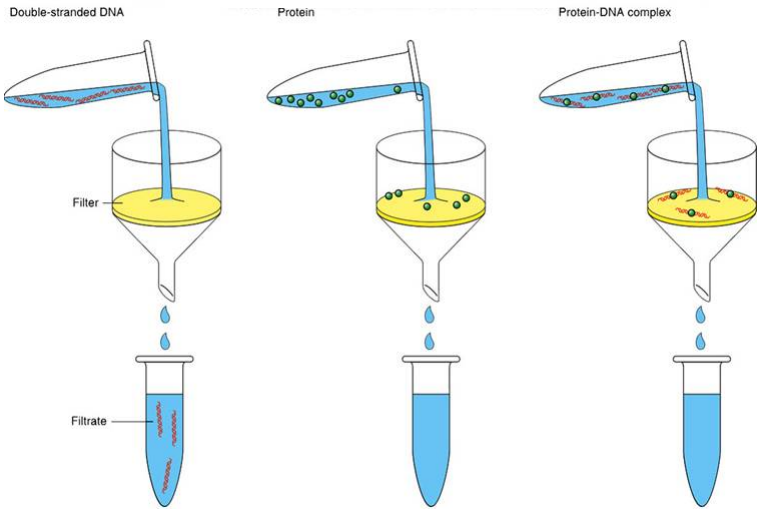
Unión a filtros de nitrocelulosa (*filter binding*)

EMSA: *electrophoretic mobility shift assay* (*gel shift*, *band shift*, retardo en gel,etc.)

Footprinting

Yeast one hybrid system

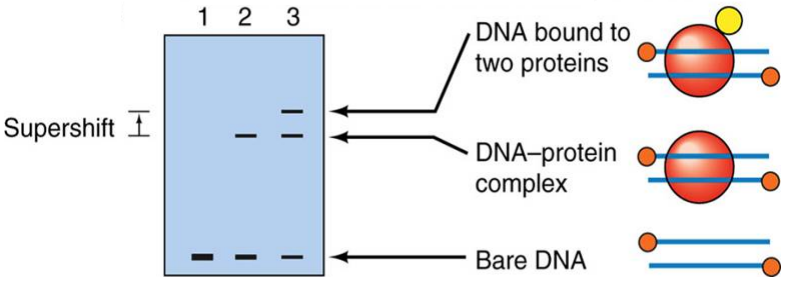
NITROCELLULOSE FILTER BINDING ASSAY



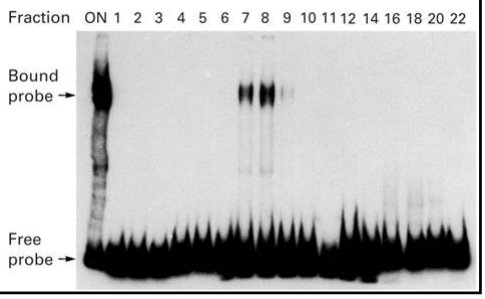
DNA alone passes through the filter while protein is retained; because of this, retained DNA must be bound to protein

WEAVER: FIG. 5.34

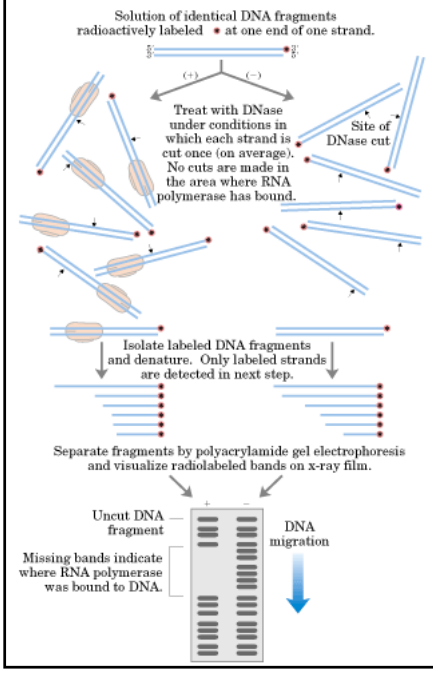
ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

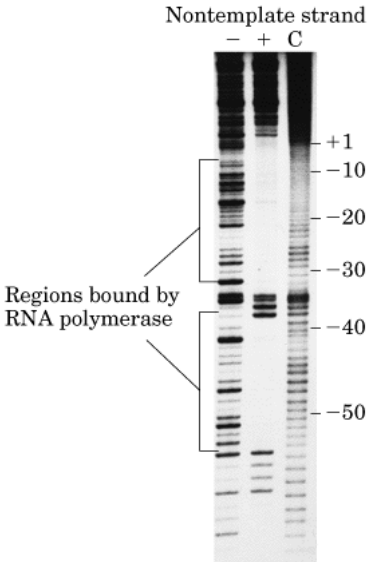


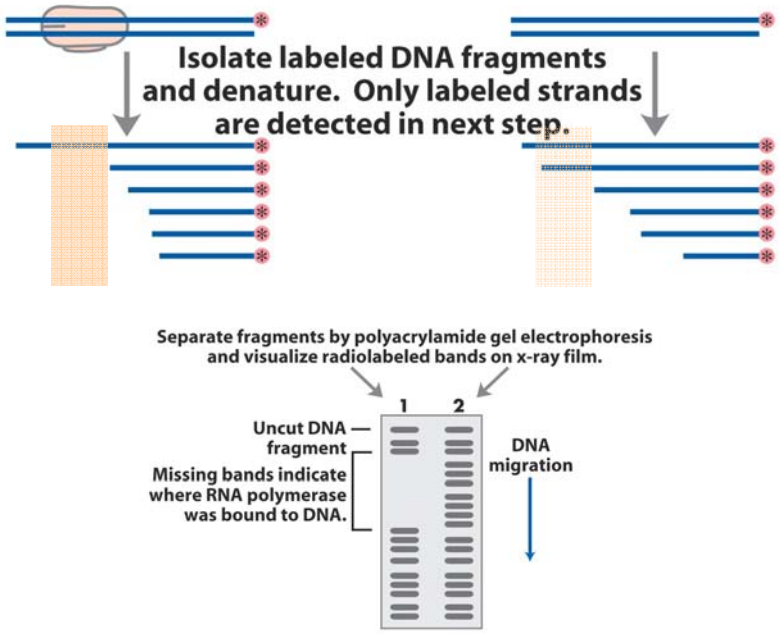
la movilidad electroforética del DNA es retrasada por la unión de la(s) **proteína**(s)



Ensayos de protección (*footprinting*)







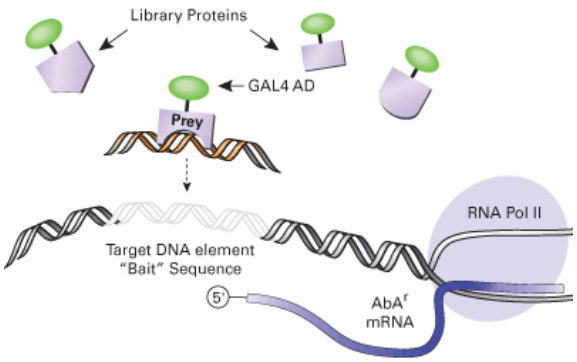
Técnicas para identificar interacciones DNA-proteína

y otras interacciones entre macromoléculas

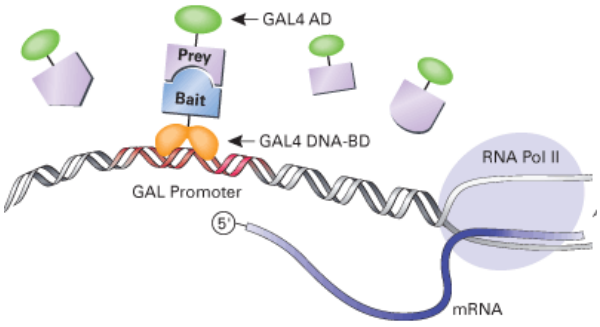
- **Yeast-one-hybrid system**
- Yeast-two-hybrid system
- Yeast-three-hybrid system

Factores de transcripción (dominios)
***Reporter genes* = genes indicadores**

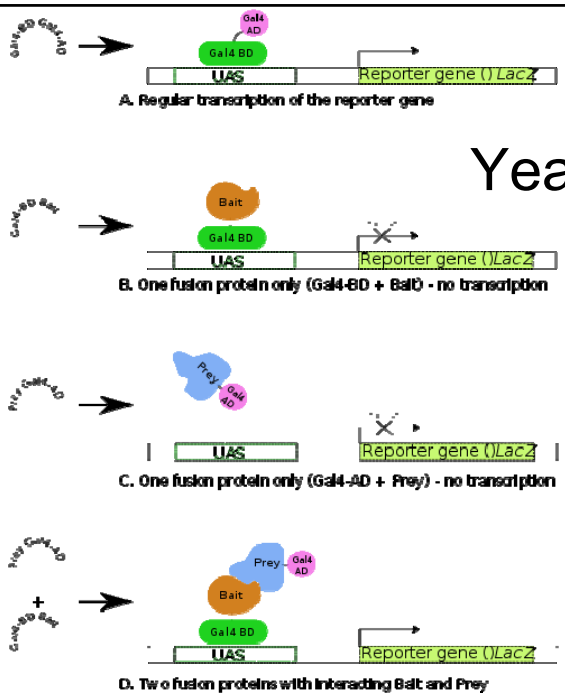
Yeast-one-hybrid



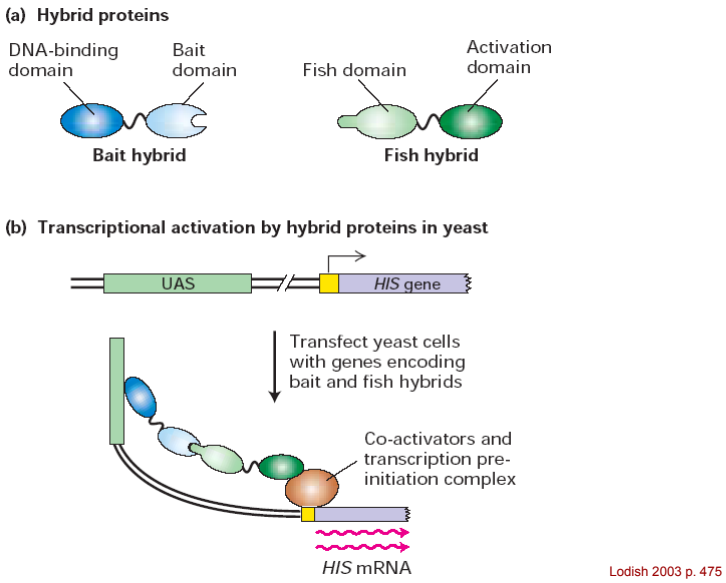
Yeast-two-hybrid



Yeast-two-hybrid

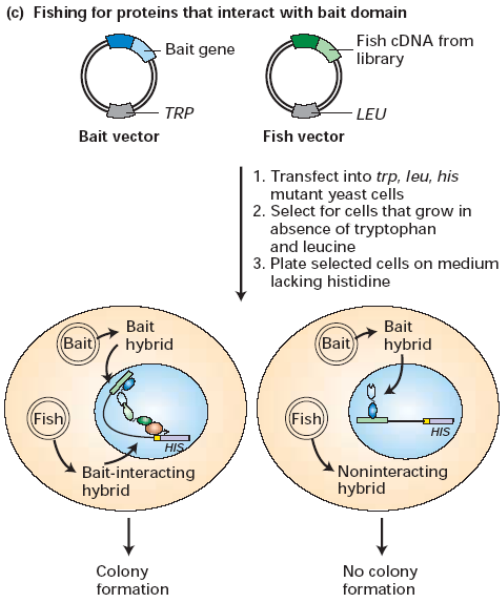


Yeast two hybrid system

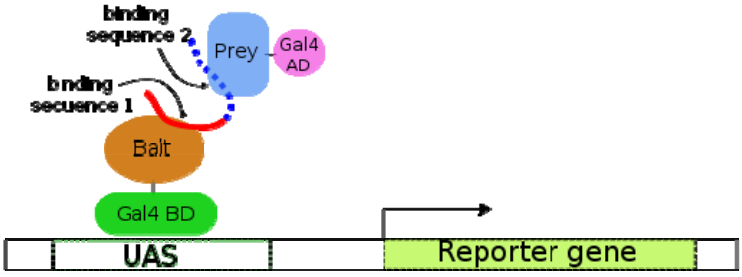


Lodish 2003 p. 475

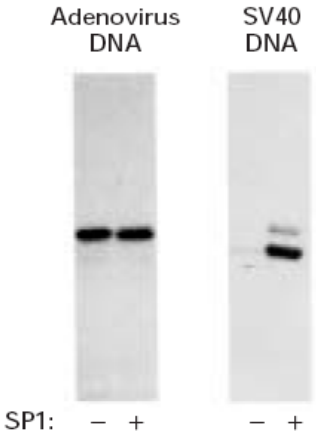
Yeast two hybrid system



Yeast-three-hybrid



Estudio de actividad de promotores y factores de transcripción



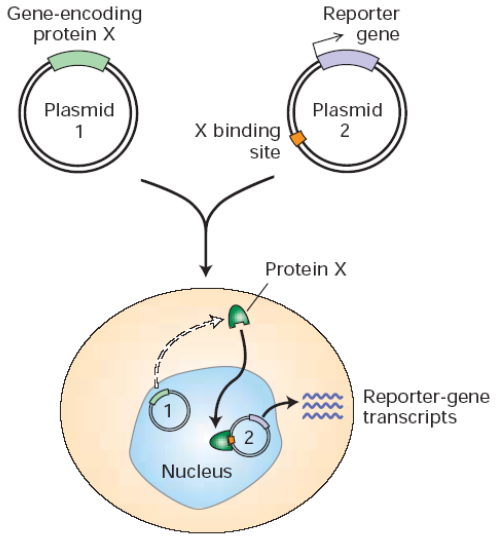
EXPERIMENTAL FIGURE 11-15
Transcription factors can be identified by *in vitro* assay for transcription activity.
SP1 was identified based on its ability to bind to a region of the SV40 genome that contains six copies of a GC-rich promoter-proximal element and was purified by column chromatography. To test the transcription-activating ability of purified SP1, it was incubated in vitro with template DNA, a protein fraction containing RNA polymerase II and associated general transcription factors, and labeled ribonucleoside triphosphates. The labeled RNA products were subjected to electrophoresis and autoradiography. Shown here are autoradiograms from assays with adenovirus and SV40 DNA in the absence (-) and presence (+) of SP1. SP1 had no significant effect on transcription from the adenovirus promoter, which contains no SP1-binding sites. In contrast, SP1 stimulated transcription from the SV40 promoter about tenfold. [Adapted from M. R. Briggs et al., 1986, *Science* **234**:47.]

Lodish 2003, p.455

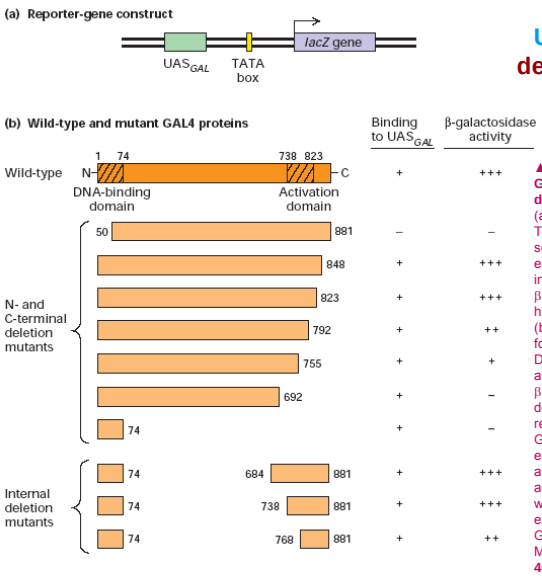
▲ **EXPERIMENTAL FIGURE 11-16**

In vivo transfection assay measures transcription activity to evaluate proteins believed to be transcription factors.

The assay system requires two plasmids. One plasmid contains the gene encoding the putative transcription factor (protein X). The second plasmid contains a reporter gene (e.g., *lacZ*) and one or more binding sites for protein X. Both plasmids are simultaneously introduced into cells that lack the gene encoding protein X. The production of reporter-gene RNA transcripts is measured; alternatively, the activity of the encoded protein can be assayed. If reporter-gene transcription is greater in the presence of the X-encoding plasmid, then the protein is an activator; if transcription is less, then it is a repressor. By use of plasmids encoding a mutated or rearranged transcription factor, important domains of the protein can be identified.

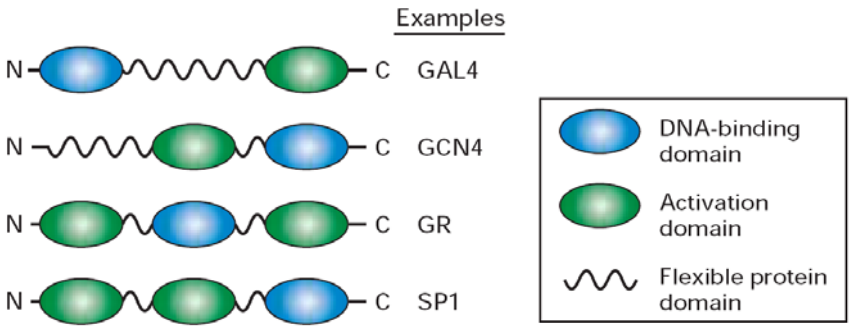


Deletion mutants of the GAL4 gene in yeast with a UAS*GAL* reporter-gene construct demonstrate the separate functional domains in an activator.



▲ **EXPERIMENTAL FIGURE 11-17 Deletion mutants of the GAL4 gene in yeast with a UAS*GAL* reporter-gene construct demonstrate the separate functional domains in an activator.** (a) Diagram of DNA construct containing a *lacZ* reporter gene and TATA box ligated to UAS*GAL*, a regulatory element that contains several GAL4-binding sites. The reporter-gene construct and DNA encoding wild-type or mutant (deleted) GAL4 were simultaneously introduced into mutant (*gal4*) yeast cells, and the activity of β-galactosidase expressed from *lacZ* was assayed. Activity will be high if the introduced *GAL4* DNA encodes a functional protein. (b) Schematic diagrams of wild-type GAL4 and various mutant forms. Small numbers refer to positions in the wild-type sequence. Deletion of 50 amino acids from the N-terminal end destroyed the ability of GAL4 to bind to UAS*GAL* and to stimulate expression of β-galactosidase from the reporter gene. Proteins with extensive deletions from the C-terminal end still bound to UAS*GAL*. These results localize the DNA-binding domain to the N-terminal end of GAL4. The ability to activate β-galactosidase expression was not entirely eliminated unless somewhere between 126–189 or more amino acids were deleted from the C-terminal end. Thus the activation domain lies in the C-terminal region of GAL4. Proteins with internal deletions (*bottom*) also were able to stimulate expression of -galactosidase, indicating that the central region of GAL4 is not crucial for its function in this assay. [See J. Ma and M. Ptashne, 1987, *Cell* **48**:847; I. A. Hope and K. Struhl, 1986, *Cell* **46**:885; and R. Brent and M. Ptashne, 1985, *Cell* **43**:729.]

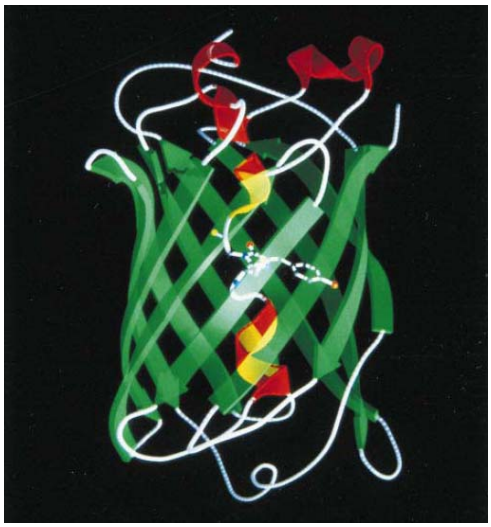
modular structure of eukaryotic transcription activators



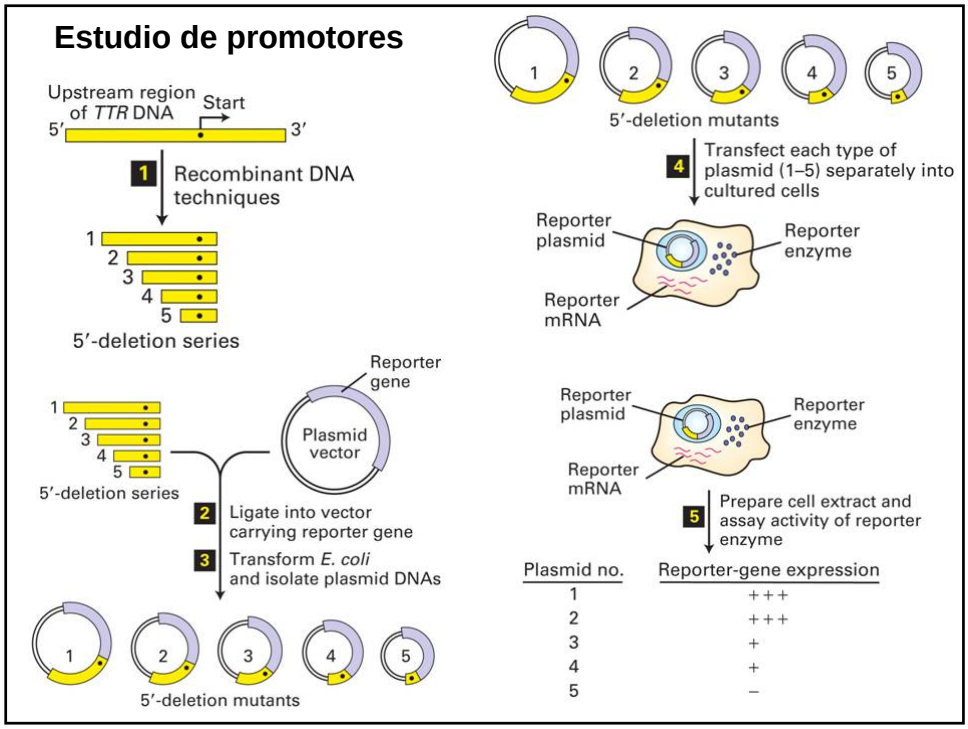
Schematic diagrams illustrating the modular structure of eukaryotic transcription activators.
These transcription factors may contain more than one **activation domain** (**AD**) but rarely contain more than one **DNA-binding domain** (**DBD**).
GAL4 and GCN4 are yeast transcription activators. The glucocorticoid receptor (GR) promotes transcription of target genes when certain hormones are bound to the C-terminal activation domain. SP1 binds to GC-rich promoter elements in a large number of mammalian genes.

Uso de "reporter genes" o genes indicadores

GFP



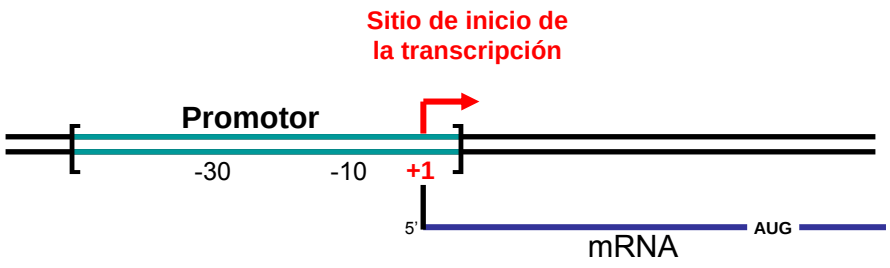
Estudio de actividad de promotores



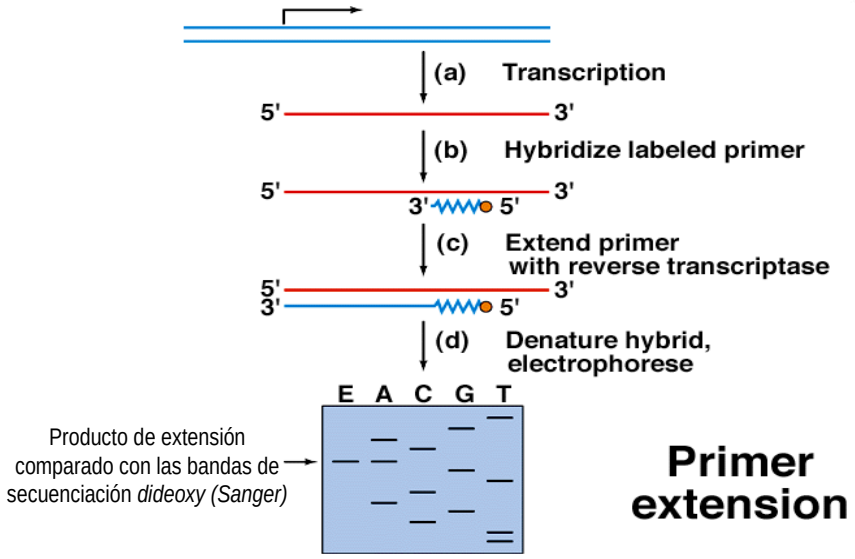
Identificación del inicio de la transcripción

Primer extension
Race: *rapid amplification of cDNA ends*
Mapeo con S1
Run off

¿Cómo se determina el sitio de inicio de la transcripción?



Determinación del sitio de inicio de la transcripción



WEAVER: FIG. 5.29

Mapeo de extremo 5´ mediante extensión de cebador (*primer extension*)