Aplikovaná chemie a biochemie

Přednáška č. 2

Proteinové techniky
(1)

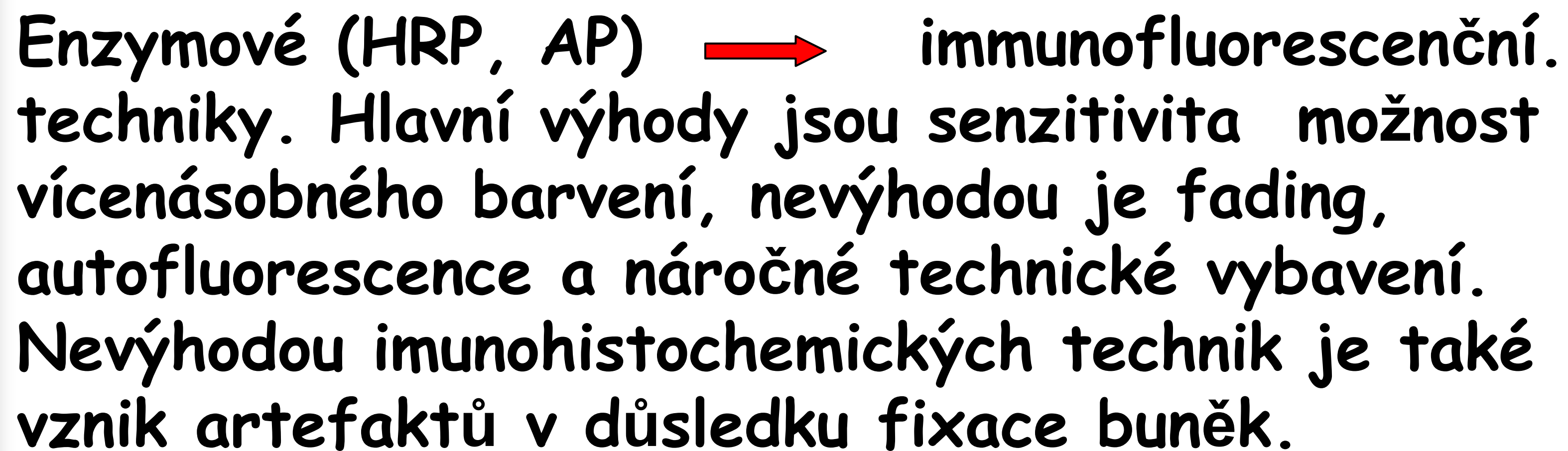
- lokalizace v buňce (live cell imaging; imunofluorescenční techniky; frakcionace);

- interakce mezi proteiny (imunoprecipitace; GST-pull-down; yeast two-hybrid screen);

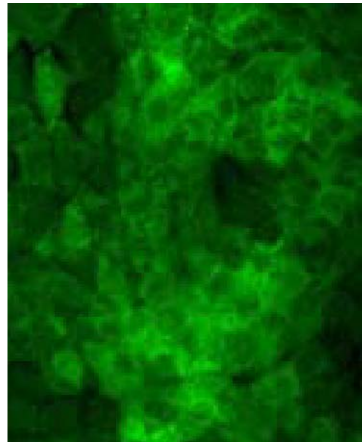
- interakce s DNA; detekce transkripční aktivity

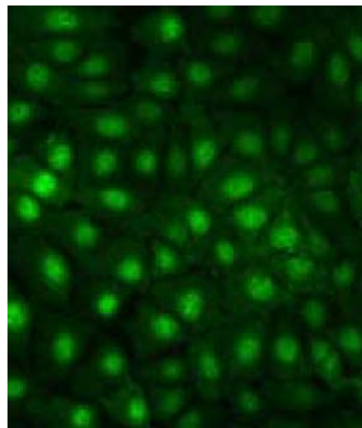
• imunodetekce – imunofluorescenční a imunohistochemické techniky; kolokalizace proteinů – konfokální mikroskopie;

Marker dané organely (subcel. komp.), negativní kontrola.

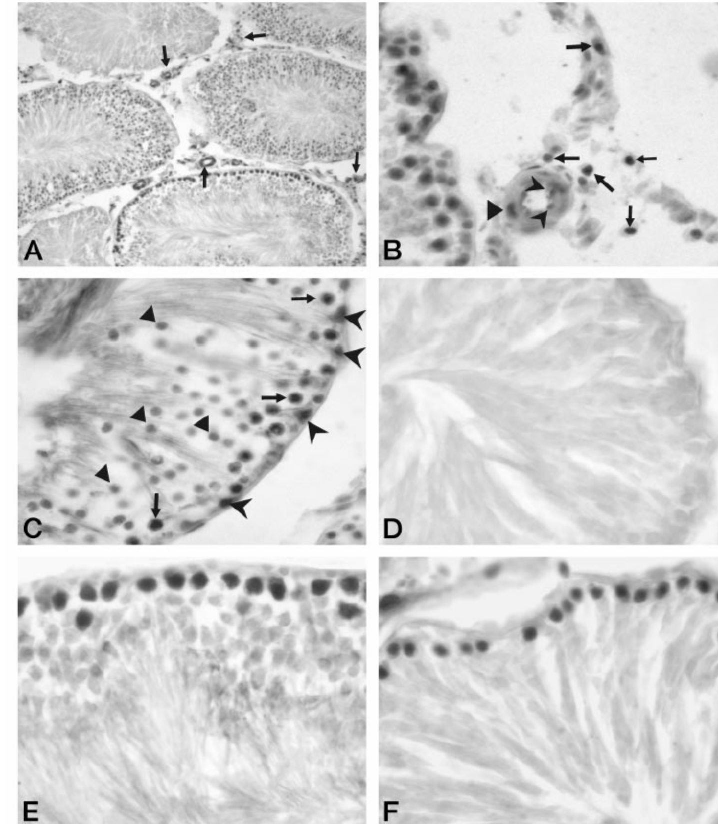


Příklad: Lokalizace Ah receptoru v buňce





+ TCDD;
30 min



AhR ve buněčné linii WB-F344:

sekundární protilátka značená FITC

AhR ve varlatech potkana:

sekundární protilátka značená HRP

- live cell imaging – využití proteinů značených např. fluorescenčními proteiny – time-lapse video, FLIP, FRET;

Výhoda – možnost pracovat s živými buňkami – je možné sledovat translokace proteinů, pohyby organel, pohyb proteinů v rámci buňky, stanovit přibližné relativní množství proteinu i proteinové interakce (intermolecular FRET).

Fluorescenční markery:

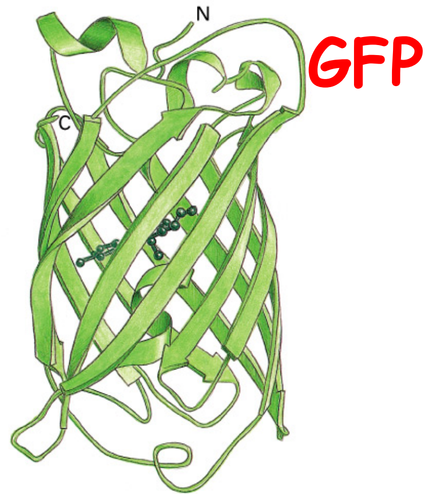


Figure 9–43. Molecular Biology of the Cell, 4th Edition.

Fluorescence can be used to visualize cell structures at many levels. Originally, fluorescence was mainly observed from small organic dyes attached by means of antibodies to the protein of interest. However, antibody targeting of intracellular proteins normally requires cell fixation and permeabilization.

Later, fluorophores could directly recognize organelles, nucleic acids, and certain important ions in living cells. In the past decade, fluorescent proteins have enabled noninvasive imaging in living cells and organisms of reporter gene expression, protein trafficking, and many dynamic biochemical signals.

Fluorescenční markery:

Table 1. Applications of fluorophores in protein detection. Applicability ranges from most optimal (++) to generally not applicable (–), and (+/–) indicates applicable in some cases.

Fluorophores for examination of	Small organic dyes (antibody-targeted)	Quantum dots (antibody-targeted)	Fluorescent proteins	Genetic tags with small dyes
Endogenous proteins	++	+	–	–
Clinical specimens	++	+	–	–
Animals	Ex vivo	Ex vivo	Transgene live	Transgene ex vivo
Primary tissues	++	+	Transgene/virus	Transgene/virus
Live cells in culture	Surface	Surface	++	+
Multiple proteins at once	++	++	++	–
Dynamic interactions	+/–	+/–	++	Combination with FP
Turnover/synthesis	–	–	+	+
Activation state	Phospho-specific	Phospho-specific	FRET sensors	Combination with FP
CALI	+	–	+	++
EM	+/–	++	+/–	+
Protein microarrays	++	+	–	–
In gel fluorescence	–	–	+	+
Western blot	–	+	–	–
Major advantages	Diversity of properties	Bright and photostable	Live cells and specificity	Live cells and small size
Major limitations	Targeting in live cells	Targeting and penetration	Ectopic expression	Ectopic expression, background staining
Improvements expected	Generic conjugated primary antibodies	Smaller, diversity of properties	Better properties, generic sensors	New applications

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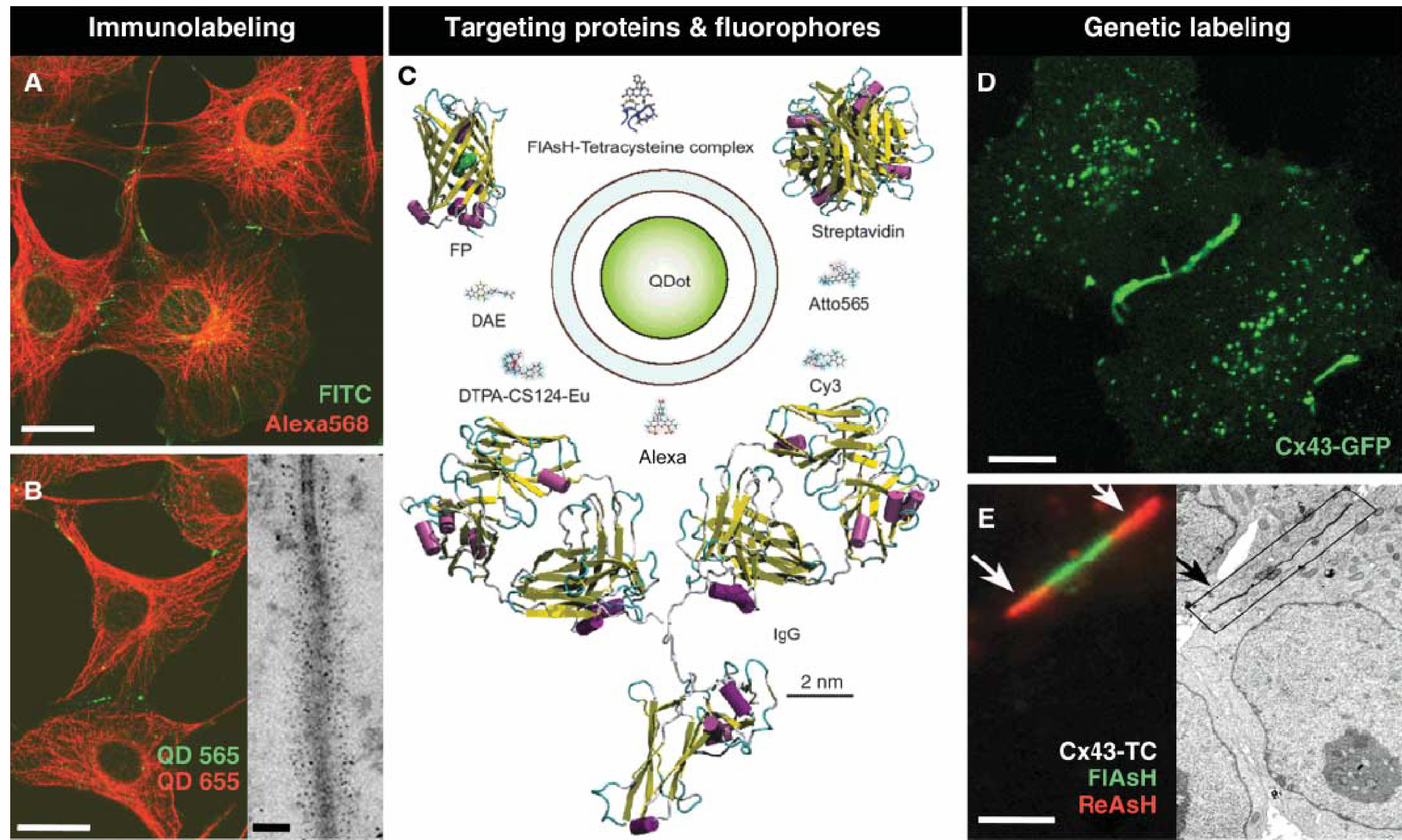
Fluorescenční markery:



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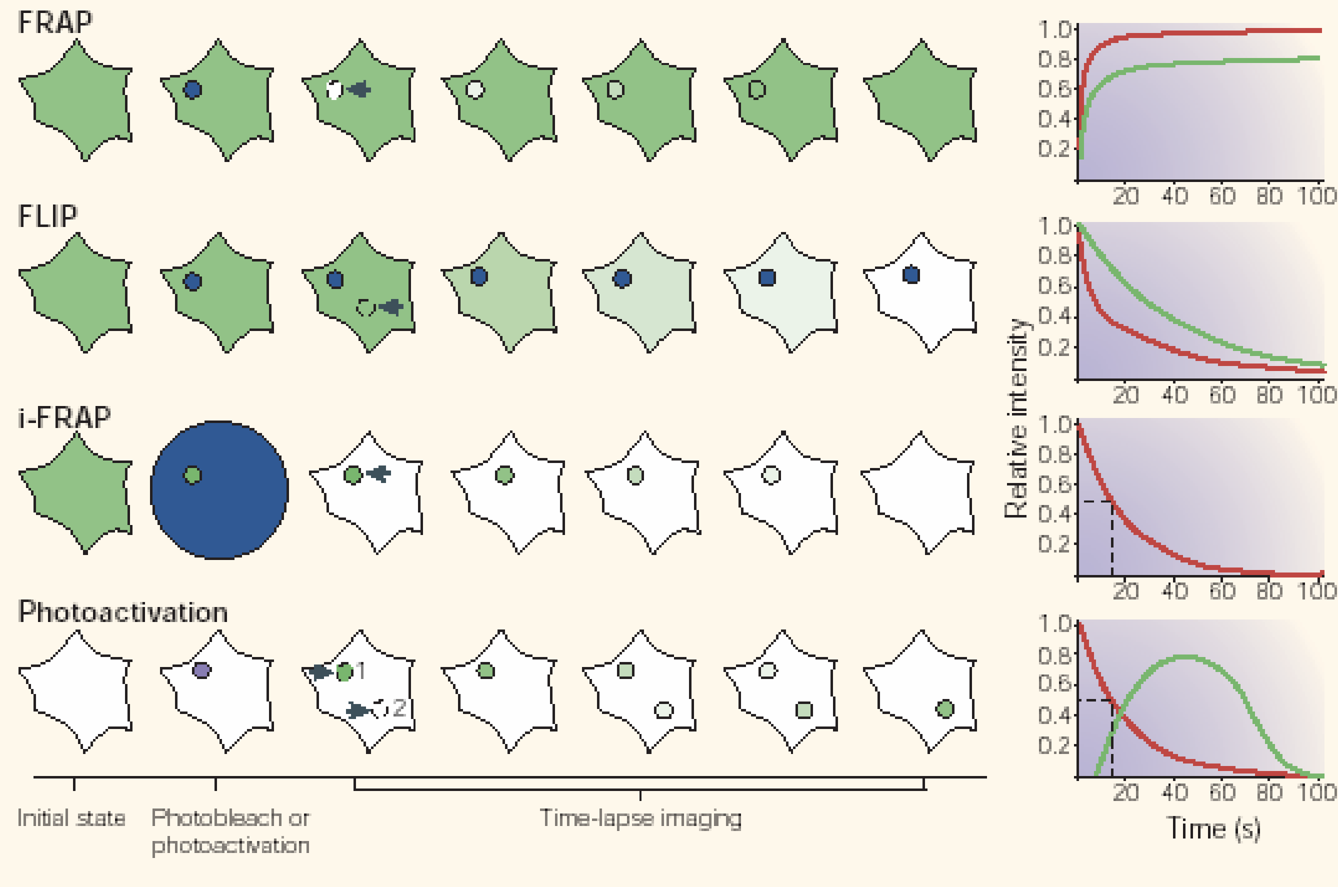
Fluorescenční markery:

Table 1 | Properties of the best FP variants[a,b]

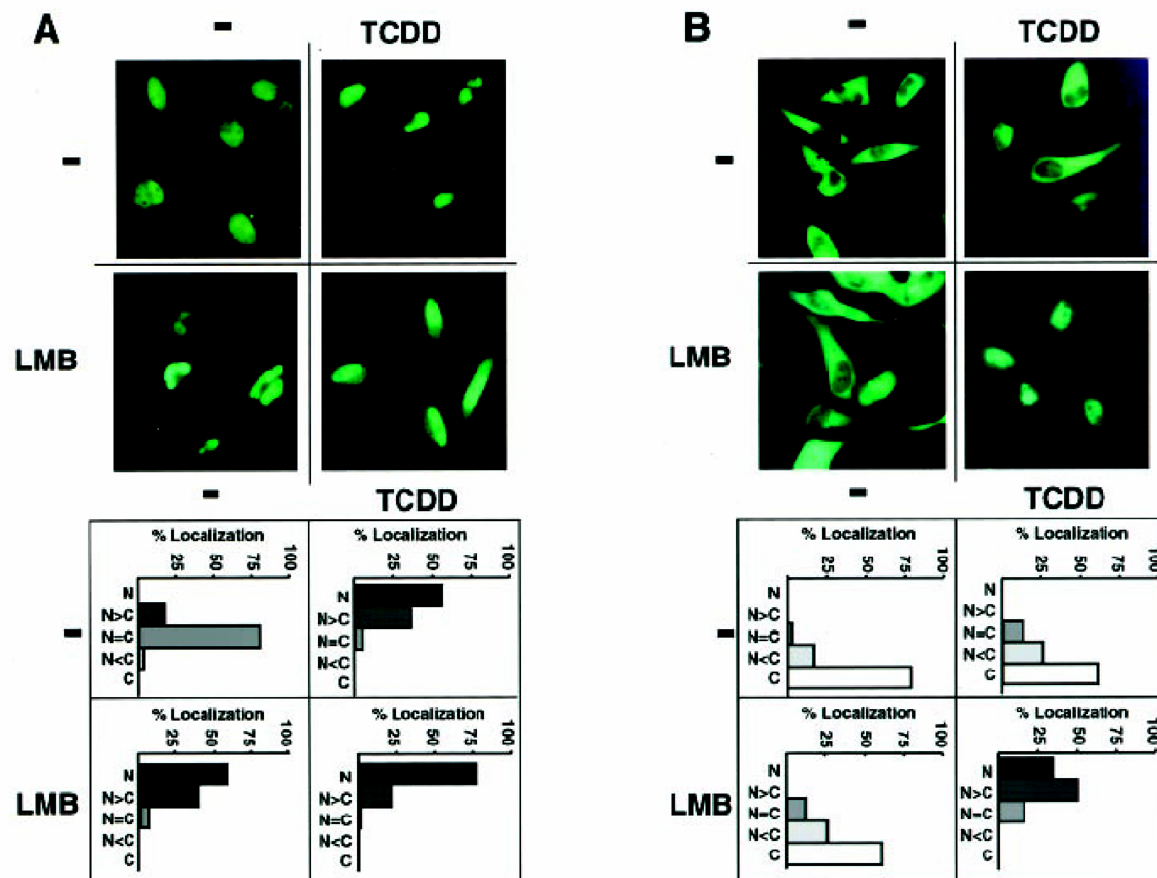
Class	Protein	Source laboratory (references)	Excitation[c] (nm)	Emission[d] (nm)	Brightness[e]	Photostability[f]	pKa	Oligomerization
Far-red	mPlum[g]	Tsien (5)	590	649	4.1	53	<4.5	Monomer
Red	mCherry[g]	Tsien (4)	587	610	16	96	<4.5	Monomer
	tdTomato[g]	Tsien (4)	554	581	95	98	4.7	Tandem dimer
	mStrawberry[g]	Tsien (4)	574	596	26	15	<4.5	Monomer
	J-Red[h]	Evrogen	584	610	8.8*	13	5.0	Dimer
	DsRed-monomer[h]	Clontech	556	586	3.5	16	4.5	Monomer
Orange	mOrange[g]	Tsien (4)	548	562	49	9.0	6.5	Monomer
	mKO	MBL Intl. (10)	548	559	31*	122	5.0	Monomer
Yellow-green	mCitrine[i]	Tsien (16,23)	516	529	59	49	5.7	Monomer
	Venus	Miyawaki (1)	515	528	53*	15	6.0	Weak dimer[j]
	YPet[g]	Daugherty (2)	517	530	80*	49	5.6	Weak dimer[j]
	EYFP	Invitrogen (18)	514	527	51	60	6.9	Weak dimer[j]
Green	Emerald[g]	Invitrogen (18)	487	509	39	0.69[k]	6.0	Weak dimer[j]
	EGFP	Clontech[l]	488	507	34	174	6.0	Weak dimer[j]
Cyan	CyPet	Daugherty (2)	435	477	18*	59	5.0	Weak dimer[j]
	mCFPm[m]	Tsien (23)	433	475	13	64	4.7	Monomer
	Cerulean[g]	Piston (3)	433	475	27*	36	4.7	Weak dimer[j]
UV-excitable green	T-Sapphire[g]	Griesbeck (6)	399	511	26*	25	4.9	Weak dimer[j]



Fluorescence recovery after photobleaching (FRAP)
The fluorescent signal (blue circle) is bleached in a small intracellular area and the recovery is measured inside this region as a function of time (see figure, top panel). Using this technique, diffusion coefficients (D) and the proportion of molecules that are mobile can be measured[68]. Red curve: 100% of molecules are diffusing ($D = 0.5\ \mu m^2\ s^{-1}$); green curve: 80% of molecules are diffusing ($D = 0.2\ \mu m^2\ s^{-1}$) and 20% are immobile.

Fluorescence loss in photobleaching (FLIP)
A small area (blue circle) is repeatedly photobleached and the loss in fluorescence intensity is recorded as a function of time in another region of the cell (see figure, second panel). FLIP provides measurements of the number of populations of a particular molecule and their relative proportions[69]. Green curve: a molecule that is present as a single population; red curve: two different populations of a molecule are present, each of which has a different rate constant.

Fig. 2. **Identification of an NES motif in the PAS A domain of the dioxin receptor.** HeLa cells were transfected with 500 ng of a GFP fusion construct encoding the wild type (*A*) or C216S (*B*) dioxin receptors. Following transfection, cells were treated with vehicle alone, 50 nM leptomycin B, 10 nM TCDD, or a combination of both for 2 h. Shown *below* is the statistical evaluation of 150–200 cells treated with the different chemicals. Cells were categorized as described under "Materials and Methods."



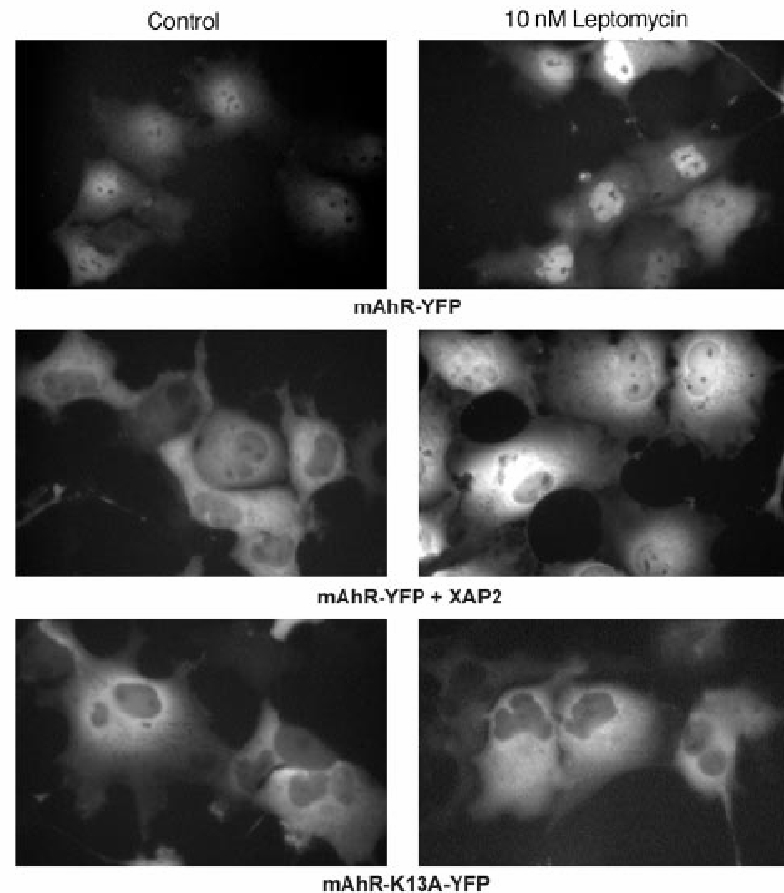


Fig. 1. **XAP2 blocks ligand-independent nucleocytoplasmic shuttling of the mAhR-YFP.** COS-1 cells grown in six-well microplates were transiently transfected with either pEYFP-mAhR, pEYFP-mAhR + pCI-XAP2, or pEYFP-mAhR-K13A. Control (carrier solvent) and 10 nM leptomycin B treated cells were visualized after 1 h of treatment by fluorescence microscopy. The mAhR-YFP alone localized throughout cells and showed nuclear retention following 1 h treatment with the export inhibitor leptomycin B. Co-expression of XAP2 resulted in cytoplasmic localization, and leptomycin B-mediated nuclear accumulation was inhibited. A mutation in the NLS abolished nuclear accumulation of the AhR-YFP in the absence of XAP2 and was unaffected by treatment with leptomycin B.

- subcelulární frakcionace následovaná WB

Výhoda – nižší spotřeba protilátek, snadné vyloučení nespecifických vazeb protilátek; nevýhoda – pracnost.

Nejčastěji používaná metoda – použití specifických detergentů, ultracentrifugace.

Sequential centrifugation:

Nuclear fraction - at 1,000g for 10 min.
Heavy mitochondrial fraction 3,000g for 10min.
Light mitochondrial fraction at 15,000-17,000g for 10 min.
Microsomal fraction at 100,000g for 45 min.

Table 1 Chemical and enzyme markers for subcellular membranes

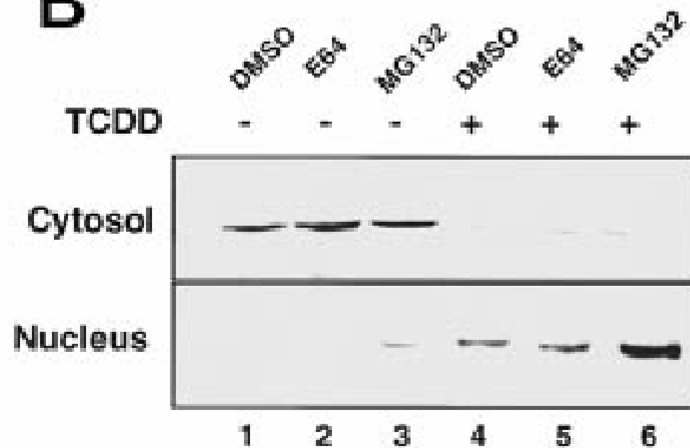
Subcellular Particle	Marker*	Comment
Nucleus	DNA	
Mitochondria	Succinate dehydrogenase	Glutamate dehydrogenase is another commonly used marker
Lysosomes	Acid phosphatase, β-galactosidase	Other hydrolytic enzymes, e.g. β-*N*-acetylglucos-aminidase, may be used
Peroxisomes	Catalase	Method using titanium oxysulphate much easier to execute than one using $KMnO_4$
Endoplasmic reticulum (ER)	NADPH-cytochrome c reductase, rotenone-insensitive NADH-cytochrome c reductase	Glucose-6-phosphatase is only a reliable marker for liver and kidney ER.
Rough ER	As ER plus RNA	
Golgi	UDP-galactose galactosyl transferase	Method using ovalbumin as galactose acceptor is easiest to execute. Properly a marker only for the *trans*-Golgi.
Plasma membrane	5'-nucleotidase, Na^+/K^+-ATPase, leucine aminopeptidase, alkaline phosphatase	Only 5'-nucleotidase and alkaline phosphatase are reliable markers for cultured cells. Plasma membrane domain specificity of these markers is common in morphologically polarized cells.

Composition of the pellets

The **Nuclear Pellet** contains, in addition to nuclei, mitochondria, sheets of plasma membrane (if present) and, if the homogenate has not been filtered, unbroken cells and debris (including connective tissue). Formation of this pellet is sometimes carried out at 500g rather than 1000g.
The **Heavy Mitochondrial Pellet** contains predominantly, mitochondria with rather few contaminants and is a common source of these organelles for respiratory studies. Minor components such as lysosomes, peroxisomes, Golgi membranes and various membrane vesicles are present largely because of entrapment during the pelleting process. Some plasma membrane fragments may also be present. These contaminants can be reduced by repeated washing.
The **Light Mitochondrial Pellet** contains mitochondria, lysosomes, peroxisomes, Golgi membranes and some endoplasmic reticulum. Of all differential centrifugation fractions it is the most variable in terms of the actual centrifugation parameters used: g-forces of 15-20,000g and times of 10-20 min are the most common. Some methods are designed to maintain the Golgi membranes in their "stacked" form so that they sediment at much lower g-forces (see ref 3 for more information)
The **Microsomal Pellet** is rather better defined and contains only membrane vesicles. Some of those vesicles will have been present in the cell before homogenization (e.g. endosomes, secretory vesicles and vesicles from the *trans*-Golgi network), others from the plasma membrane, Golgi and smooth and rough endoplasmic reticulum, will have been produced by the homogenization procedure.

A

B



Characterization of DR depletion in Hepa1c1c7 cells. *A*, TCDD (10 nM) was added to Hepa1c1c7 cells for the times indicated before whole cell protein extracts were separated using 8% SDS-PAGE and analyzed by immunoblotting with anti-DR mAb. *B*, TCDD (10 nM) and/or Me2SO vehicle were added to Hepa1c1c7 cells for 2.5 h. MG132 (2.5 mM), E64 (10 mM), or vehicle (*DMSO*) were added at the same time in the lanes shown. Cytosolic and nuclear extracts were prepared and analyzed by 8% SDS-PAGE and immunoblotting using anti-DR mAb.

Interakce mezi proteiny

- stabilní – proteiny vytvářejí komplex
- tranzientní – např. interakce enzymu s proteinovým substrátem

- afinita dvou proteinů je dána rovnovážnou disociační konstantou – K_d = [A]*[B]/[AB]

Základní metody:
- GST pull-down assay
- two-hybrid methods
- co-immunoprecipitation
- FRET

is a simple technique to test interaction between a tagged protein or the bait (GST, His6, biotin ...) and another protein (test protein, or prey).

The bait protein, purified from an appropriate expression system (e.g., Escherichia coli), is immobilized on a glutathione affinity gel. The bait serves as the secondary affinity support for identifying new protein partners or for confirming a previously suspected protein partner to the bait.

Prey protein can be obtained from multiple sources including recombinant purified proteins, cell lysate or in vitro transcription/translation reactions.

Protein-protein interactions can be visualized by SDS-PAGE and associated detection methods depending on the sensitivity requirements of the interacting proteins. These methods include Coomassie or silver staining, Western blotting and [^{35}S] radioisotopic detection.

GST pull-down assay

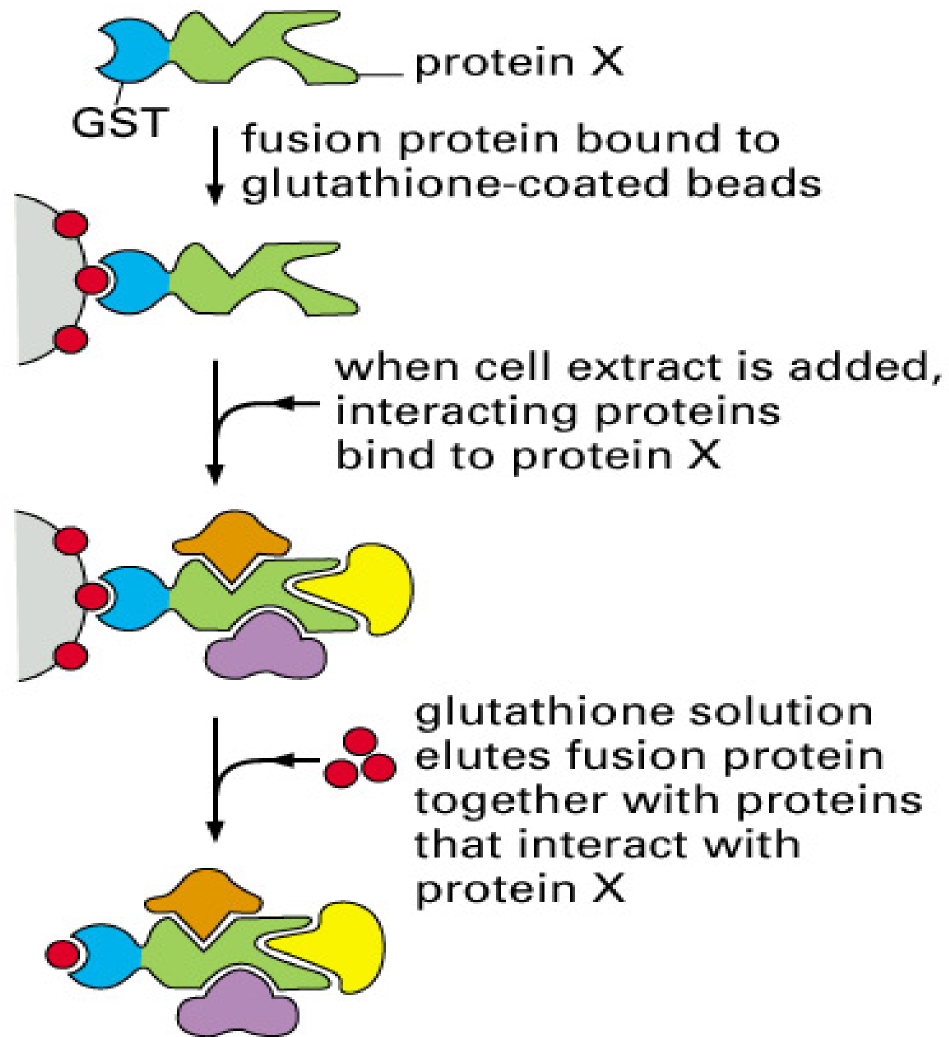
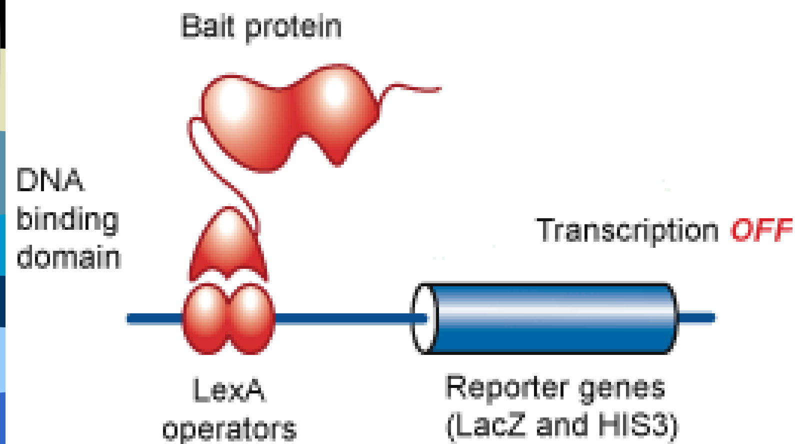
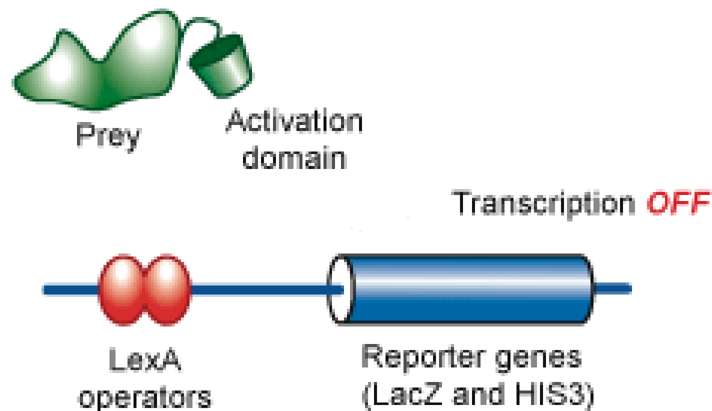


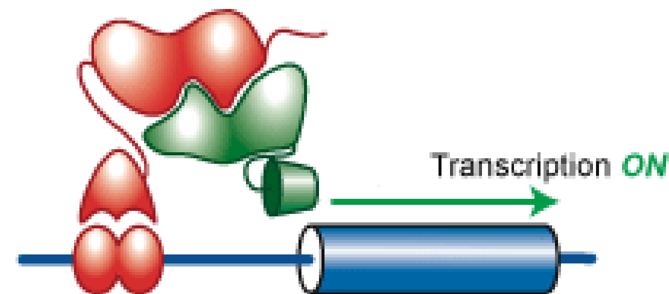
Figure 8–50. Molecular Biology of the Cell, 4th Edition.

Two-hybrid assays (interaction trap):



Two chimeras, one containing the DNA-binding domain and one that contains an activation domain, are co-transfected into an appropriate host strain. If the fusion partners (yellow and red) interact, the DB and AD are brought into proximity and can activate transcription of reporter gene.

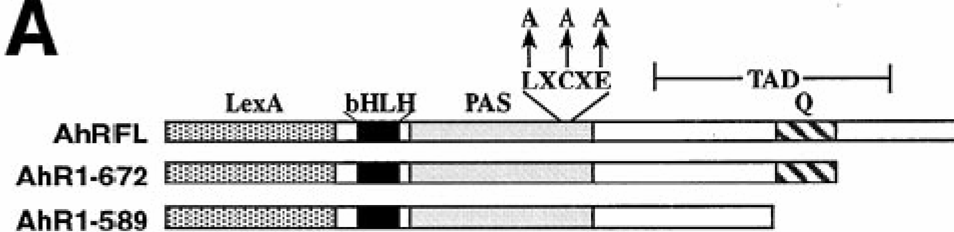


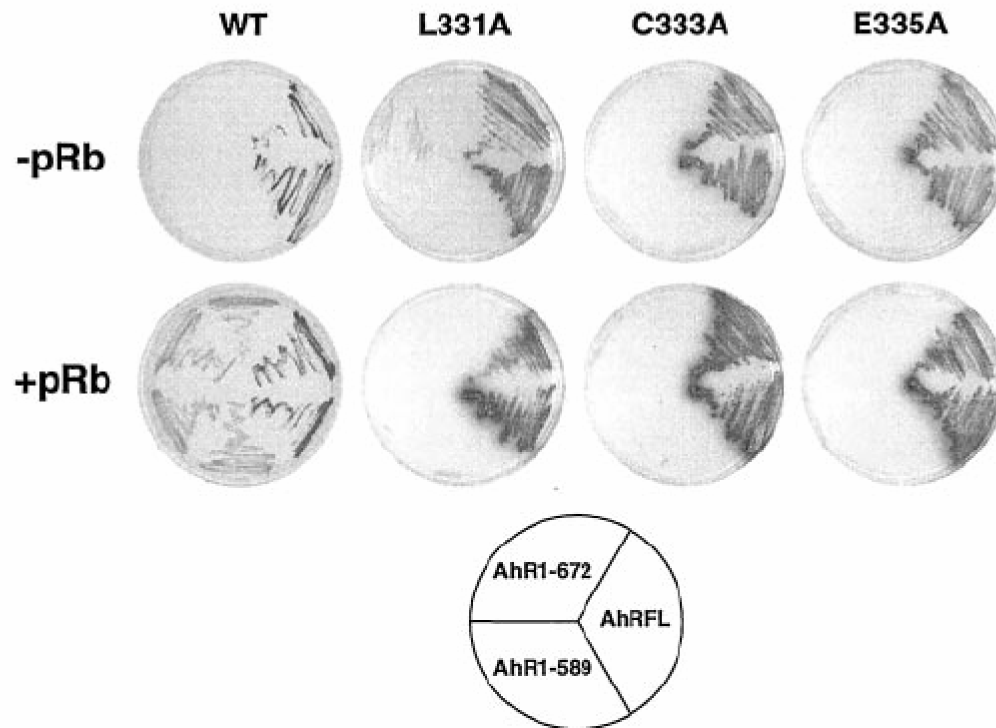


Two-hybrid assay (interaction trap):

· používají se hostitelské kmeny kvasinek (např. EGY48, EGY191) exprimující reportérový gen a dále gen pro rezistenci, např. *LEU2*, který umožňuje růst na médiu bez obsahu Leu; oba geny jsou pod kontrolou operátoru *LexA*

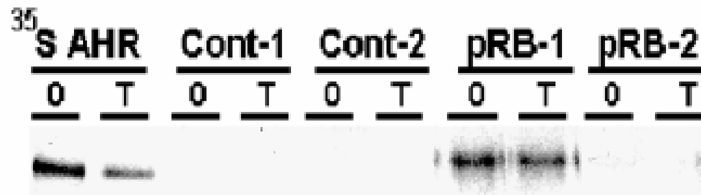
· prvním krokem je příprava „bait" proteinu fúzovaného s LexA a transformace kvasinek tímto konstruktem;
· selekce kmenů, ve kterých bait protein nepůsobí jako aktivátor, ani jako represor transkripce reportérového genu;
· příprava cDNA knihovny, nebo specifických proteinů fúzovaných s aktivační doménou (*B42* pro *LEU2* a *LacZ*);

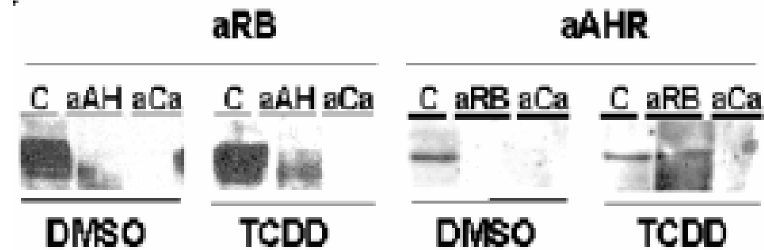




Point mutations in the LXCXE motif disrupt the AhR-pRb interaction. Site-directed mutations were introduced into the AhR to substitute each of the conserved residues in the LXCXE motif with an alanine.
Yeast cells containing a LacZ reporter plasmid (p8op-LacZ) were transformed with the wild-type (WT) or various alanine-substituted AhR bait constructs (L331A, C333A, E335A) in the absence (+pRb) and presence (-pRb) of plasmid pB42ADpRb and grown on leucine-deficient X-gal plates at 30°C for 3 to 5 days.

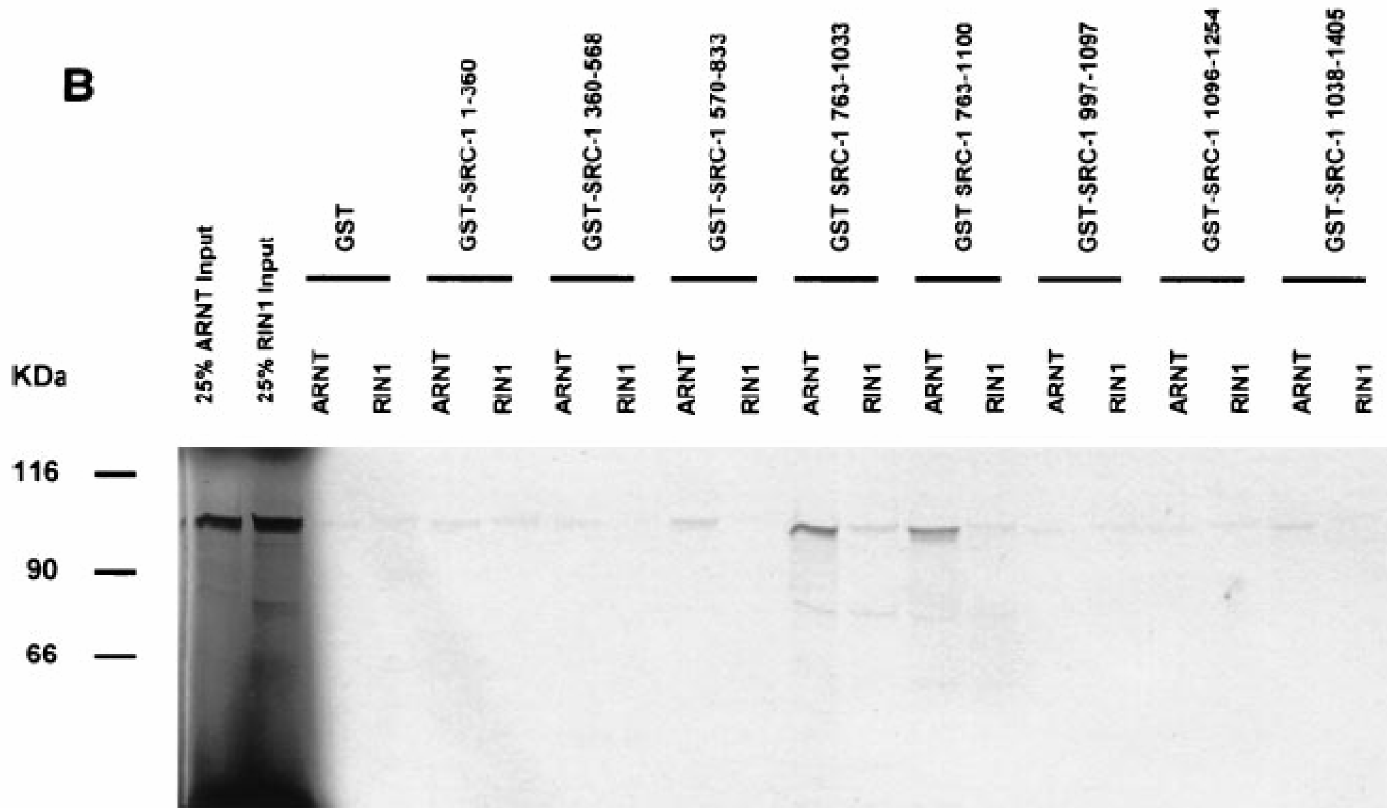
Protein-protein interactions between AHR and RB.





[^{35}S]AHR was synthesized *in vitro* in the presence (*T*) or absence (*0*) of 100 nM TCDD. Aliquots of the unfractionated translation products (*^{35}SAHR*) or of two successive effluents from control nickel-agarose columns (*Cont-1* and *Cont-2*) or RB-bearing columns (*pRB-1* and *pRB-2*) were analyzed by SDS-PAGE.

Aliquots of 1 mg each of whole cell lysates were incubated with monospecific rabbit anti-RB antiserum, anti-AHR antiserum, or control rabbit polyclonal serum. Bound proteins were recovered with protein A plus protein G-Sepharose, washed, eluted by boiling, and analyzed by SDS-PAGE and immunoblotted with anti-RB and anti-AHR antibodies



SRC-1 mutants were fused to glutathione *S*-transferase and expressed in *Escherichia coli*. Expressed fusion proteins from bacterial cell lysates were put on glutathione-agarose beads. Protein coupled to beads was incubated with in vitro-translated [^{35}S]methionine-labeled ARNT or RIN1, and absorbed proteins were subjected to SDS-PAGE. The two left lanes contained 1 μl of in vitro-translated RIN1 and 1 μl of in vitro-translated mARNT (25% input) for comparison.

FRET

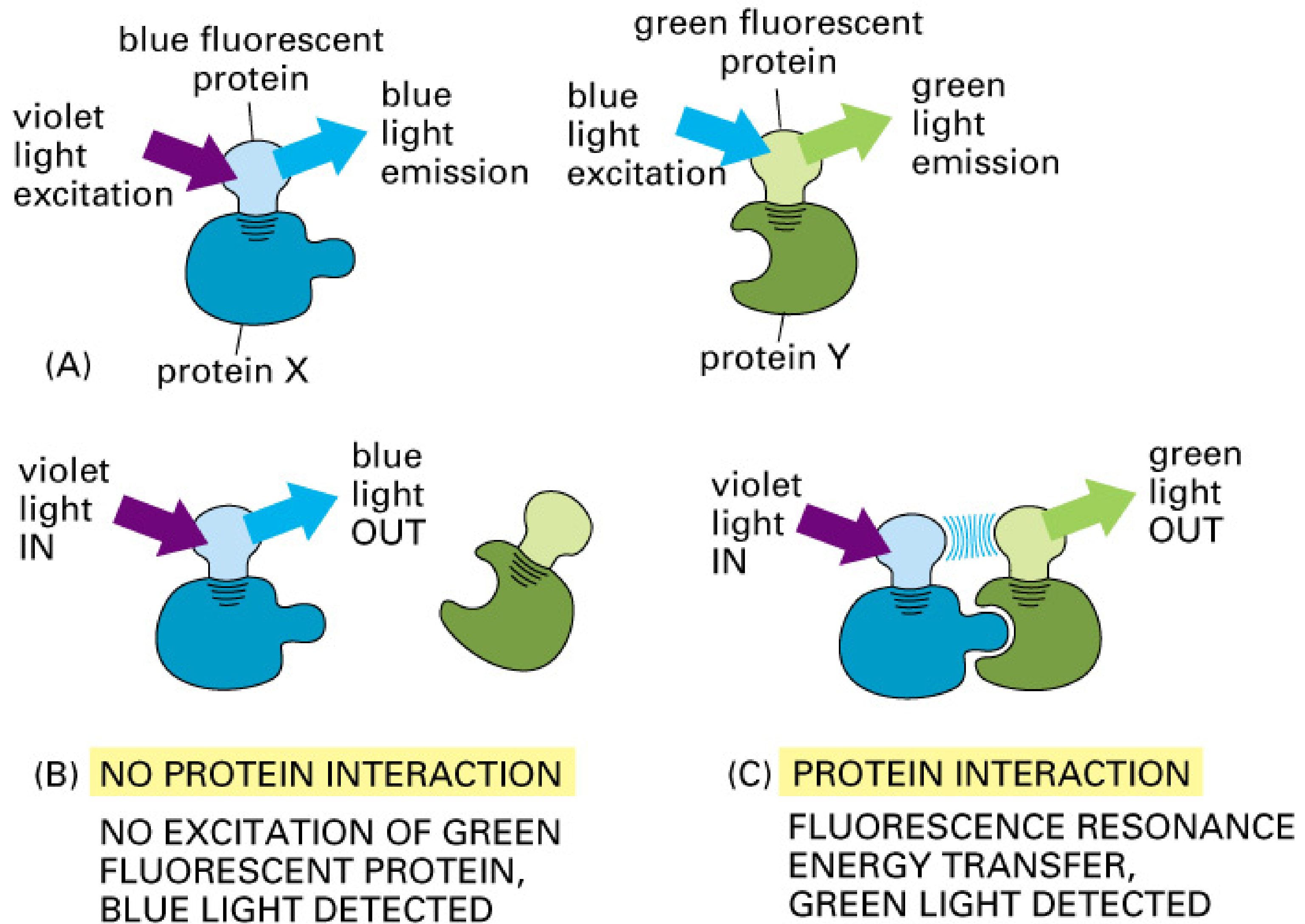
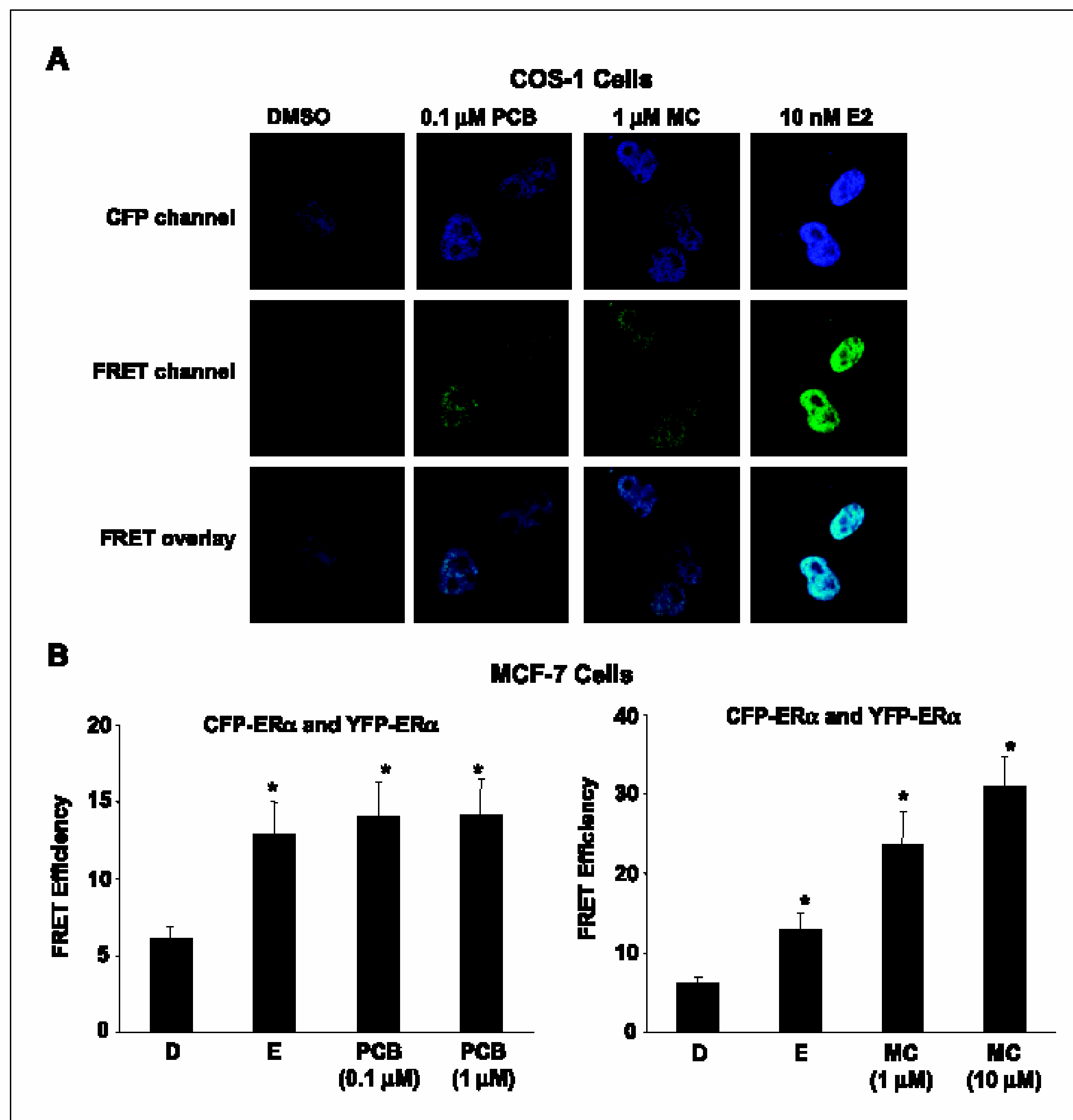


Figure 8–49. Molecular Biology of the Cell, 4th Edition.

FRET – příklad:



Analysis of ligand-induced YFP-ERα-CFP-ERα interactions in MCF-7 cells.

A, representative FRET images. Images were acquired in COS-1 cells transfected with YFP-ERα and CFP-ERα.

- příprava cytosolového nebo jaderného extraktu;

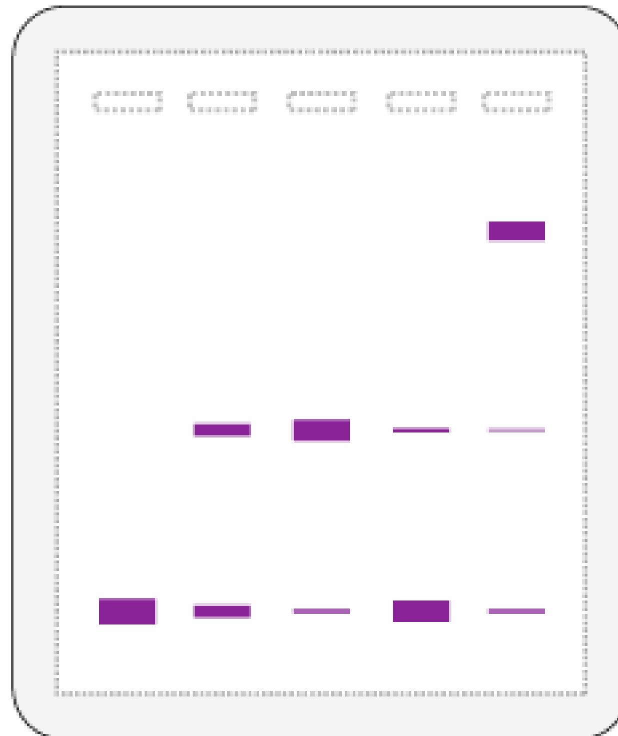
- electrophoretic mobility shift assay – EMSA (gel retardation assay);

- methylation interference; DNAse I protection assay „footprinting“

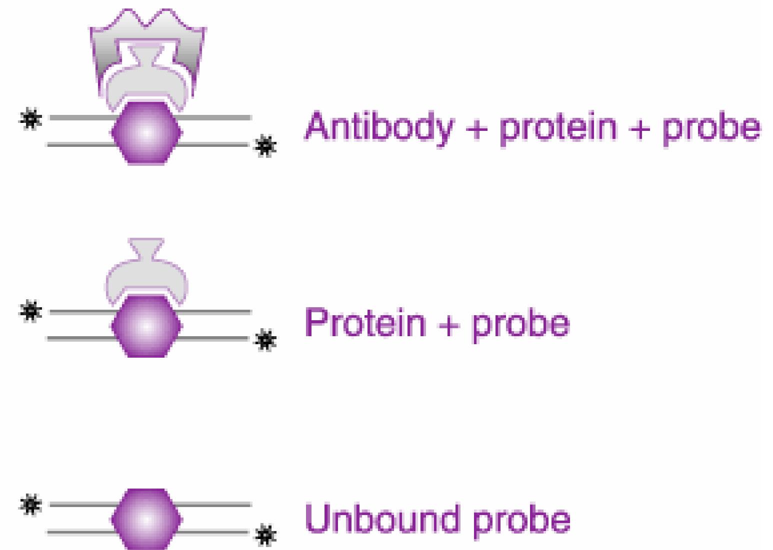
- příprava značené DNA próby (end labeling, nick translation)
- binding reaction (jaderný nebo cytosolový extrakt, carrier DNA a značené DNA próby) ⟶ loading buffer
- nedenaturující PAGE
- vysušení gelu a autoradiografie

EMSA

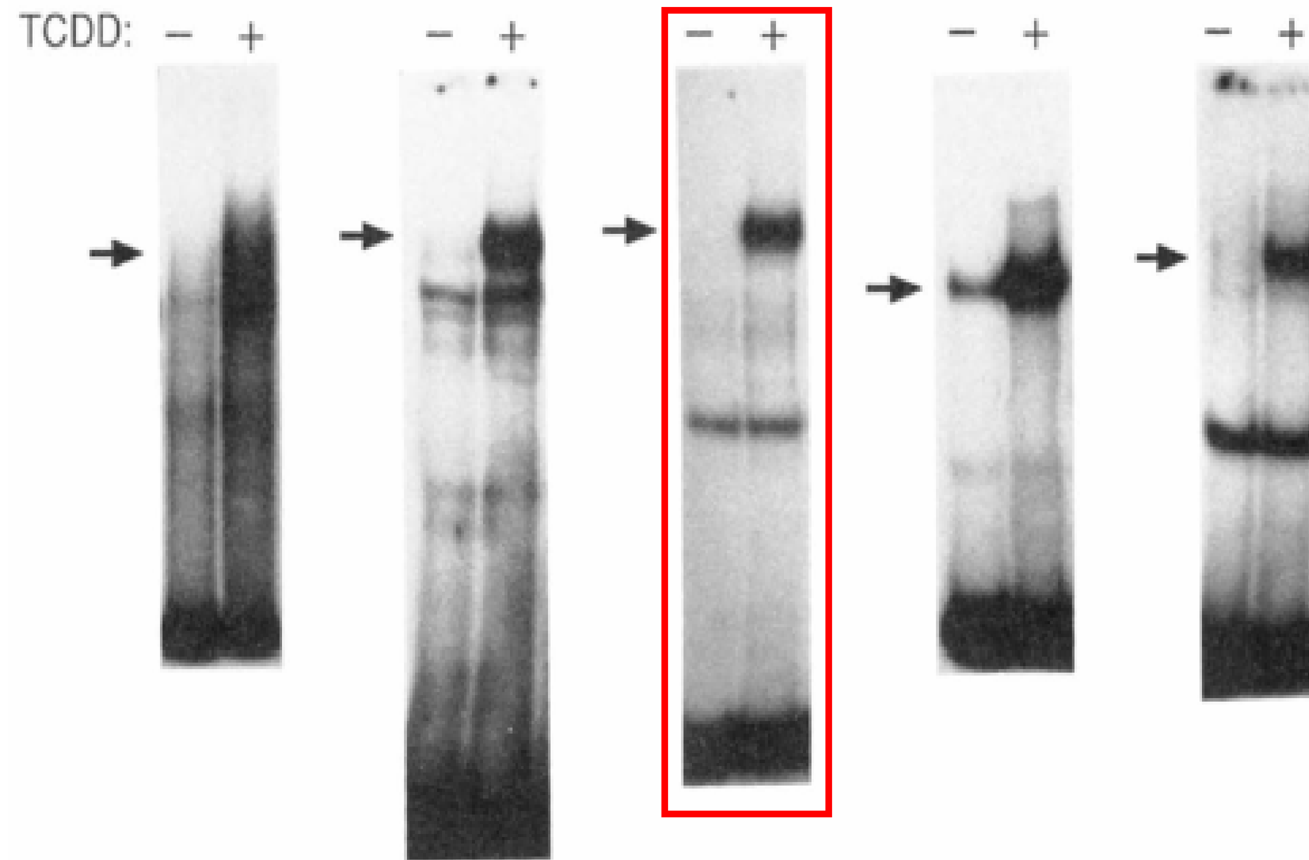
specific antibody	-	-	-	-	1x
unlabeled oligo	-	-	-	10x	-
protein	-	1x	10x	1x	10x
probe	1x	1x	1x	1x	1x



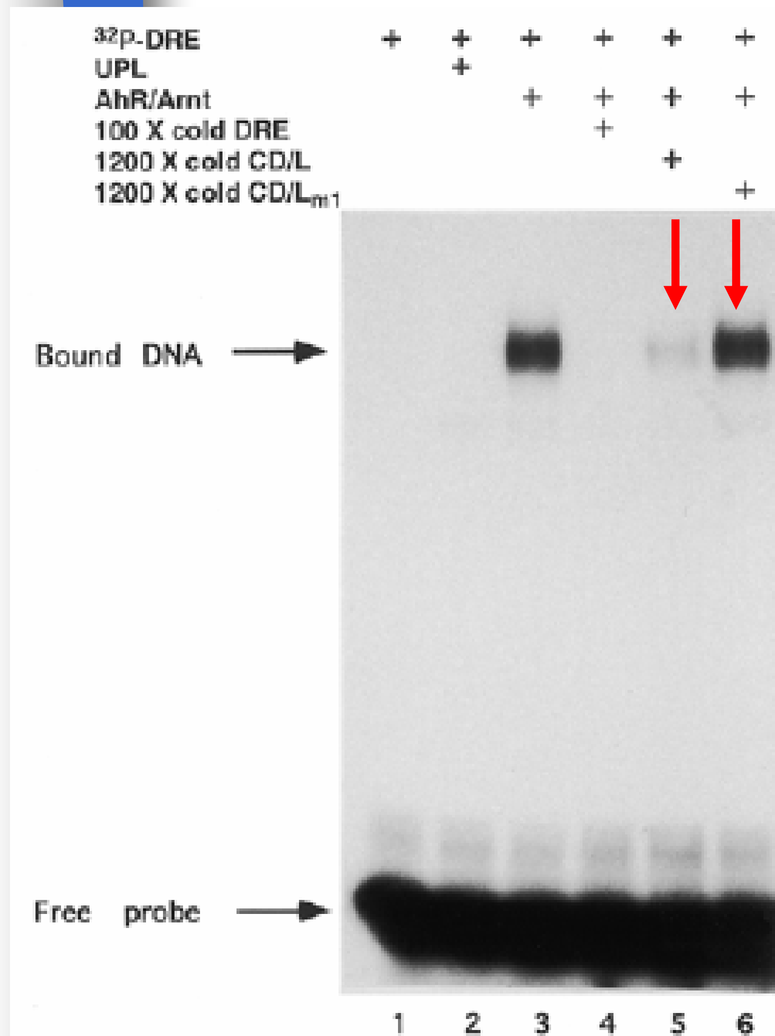
autoradiograph



EMSA



Kontrola specifity



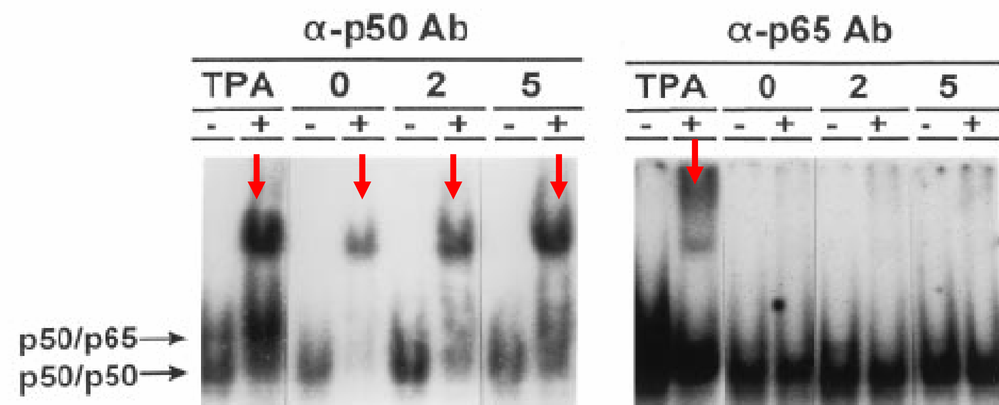


FIG. 4. Electrophoretic mobility supershift assays with specific anti-NF-κB antibodies. Hepa-1 cells were treated with 50 nM TPA for 4 hr or with 5 nM TCDD for the indicated lengths of time. Electrophoretic mobility shift assays with nuclear extracts from these cells used the NF-κB probe in Table 1 and were done in the presence (+) or absence (−) of 2 μg of anti-p50 or anti-p65 polyclonal antibodies. The *arrows* indicate the position of the p50/p50 and p50/p65 dimers.

DNase I foot printing analysis

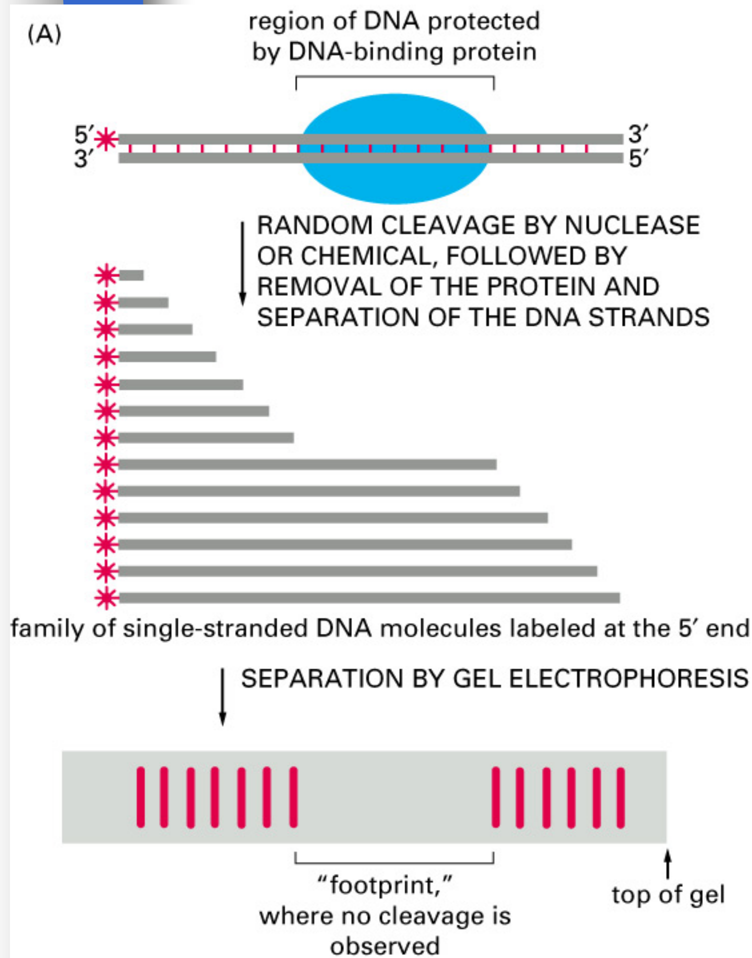
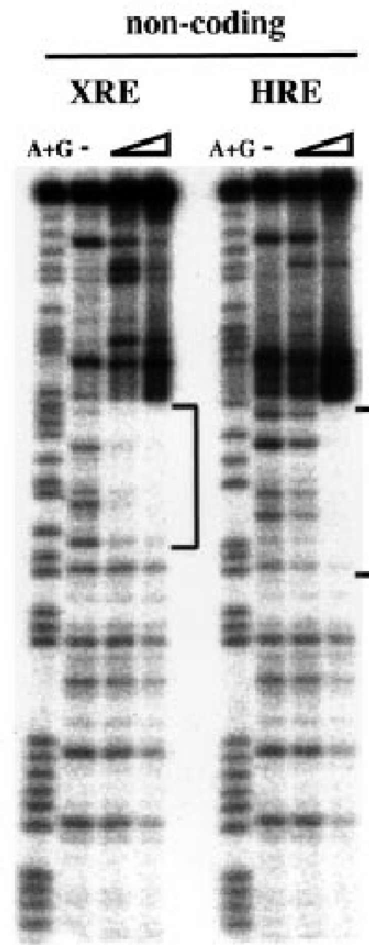
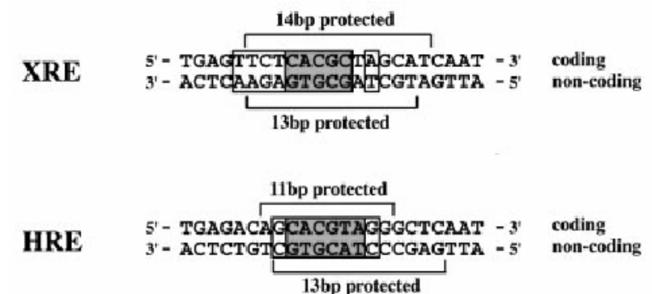


Figure 8–54 part 1 of 2. Molecular Biology of the Cell, 4th Edition.



A DNA fragment (117 bp for the coding strand and 112 bp for the non-coding strand) containing a single copy of HRE or XRE in the central part and incubated with DNase I. The digested DNA was precipitated with ethanol and separated in the denaturating acrylamide gel containing 8 M urea.



Identifikace sekvencí, na které se vážou proteiny:

- **např. XRE (DRE)** - N T/G T GCGTG A/C G/T A/T A/G N
- **genome-wide analysis**

Table 1. Sequence of bona fide DREs

Gene[a]	Site	DRE sequence 5′–3′	MS score	Reference
m*Cyp1a1*	Site a	caagctcGCGTGagaagcg	0.94	(24)
	Site b	cctgtgtGCGTGccaagca	0.95	(24)
	Site d	cggagttGCGTGagaagag	0.98	(24)
	Site e	ccagctaGCGTGacagcac	0.91	(24)
	Site f	cgggtttGCGTGcgatgag	0.97	(24)
m*Cyp1b1*	XRE5	ccccctGCGTGcggagct	0.96	(38)
r*Cyp1a1*	XRE1	cggagttGCGTGagaagag	0.98	(55)
	XRE2	gatcctaGCGTGacagcac	0.88	(55)
r*Aldh3*		cactaatGCGTGccccatc	0.85	(62)
r*Nqr1*		tcccctGCGTGcaaaggc	0.95	(63)
r*Sod1*		gaggcctGCGTGcgcgcct	0.89	(64)
r*Gstya*		gcatgttGCGTGcatccct	0.89	(48)
r*Ugt1a1*		agaatgtGCGTGacaaggt	0.92	(65)

[a]Abbreviations: m*Cyp1a1*, mouse cytochrome P4501A1; m*Cyp1b1*, mouse cytochrome P4501B1; r*Cyp1a1*, rat *Cyp1a1*; r*Aldh3*, rat aldehyde dehydrogenase-3; r*Nqr1*, rat NADPH:quinone oxidoreductase; r*Sod1*, rat Cu/Zn superoxide dismutase; *rGstYa*, rat glutathione *S*-transferase Ya; and rUgt1a1, rat UDP-glucuronosyltransferase 1A1.

! Řada těchto sekvencí neaktivuje transkripci !

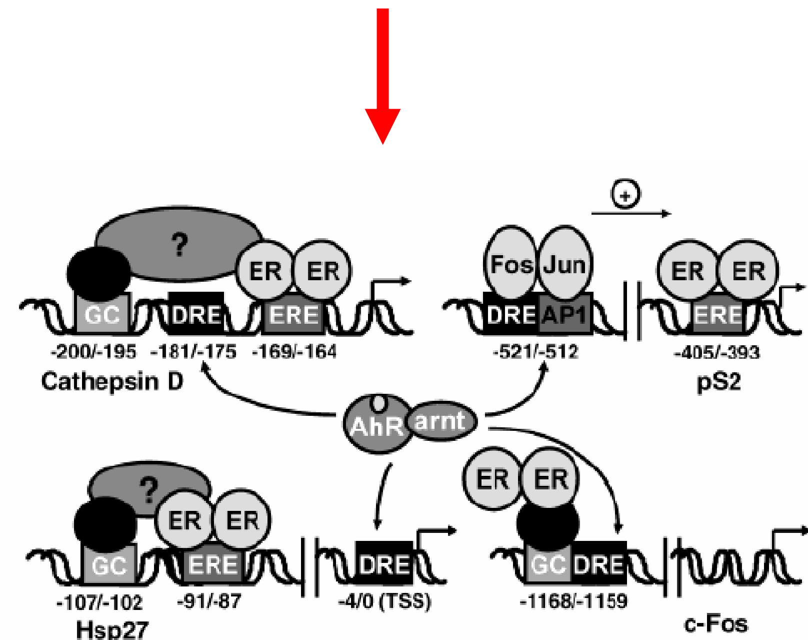
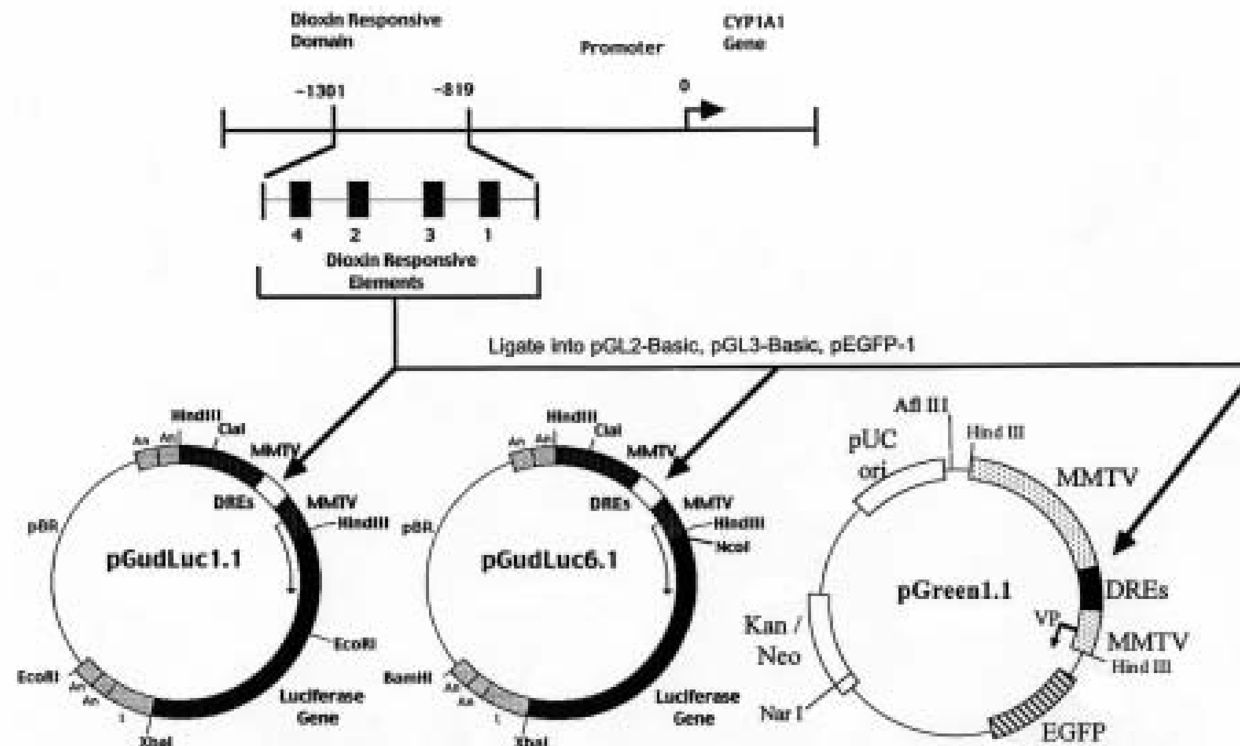


Figure 4. Functional iDREs in promoters of the cathepsin D, c-fos, heat shock protein 27, and pS2 genes (*102–107*).

- stabilní
- transientní; kontrola „transfection efficiency“ (*Renilla* luciferase, GFP, β-gal)
- důležité vlastnosti:
    - hladina proteinu = hladina mRNA
    - snadno detekovatelný produkt
    - nesmí se nacházet v cílové buňce
    - nesmí ovlivňovat cílovou buňku

Plasmid vectors



Reportérové geny:

TABLE 1. Comparison of commonly used reporter genes

Reporter gene	Advantages	Disadvantages
Chloramphenicol acetyltransferase (CAT) (bacterial)	No endogenous activity. Automated ELISA available.	Narrow linear range; use of radioisotopes; stable.
β-Galactosidase (bacterial)	Well characterised; stable; simple colorimetric readouts; sensitive bio- or chemiluminescent assays available.	Endogenous activity (mammalian cells).
Luciferase (firefly)	High specific activity; no endogenous activity; broad dynamic range; convenient assays.	Requires substrate (luciferin) and presence of O_2 and ATP.
Luciferase (bacterial)	Good for measuring/analysing prokaryotic gene transcription.	Less sensitive than firefly; not suitable for mammalian cells.
Alkaline phosphatase (human placental)	Secreted protein; inexpensive colorimetric and highly sensitive luminescent assays available.	Endogenous activity in some cells; interference with compounds being screened.
Green fluorescent protein (GFP) (jellyfish)	Autofluorescent (no substrate needed); no endogenous activity; mutants with altered spectral qualities available.	Requires post-translational modification; low sensitivity (no signal amplification).

GFP reporter

Differential interference contrast | Fluorescence

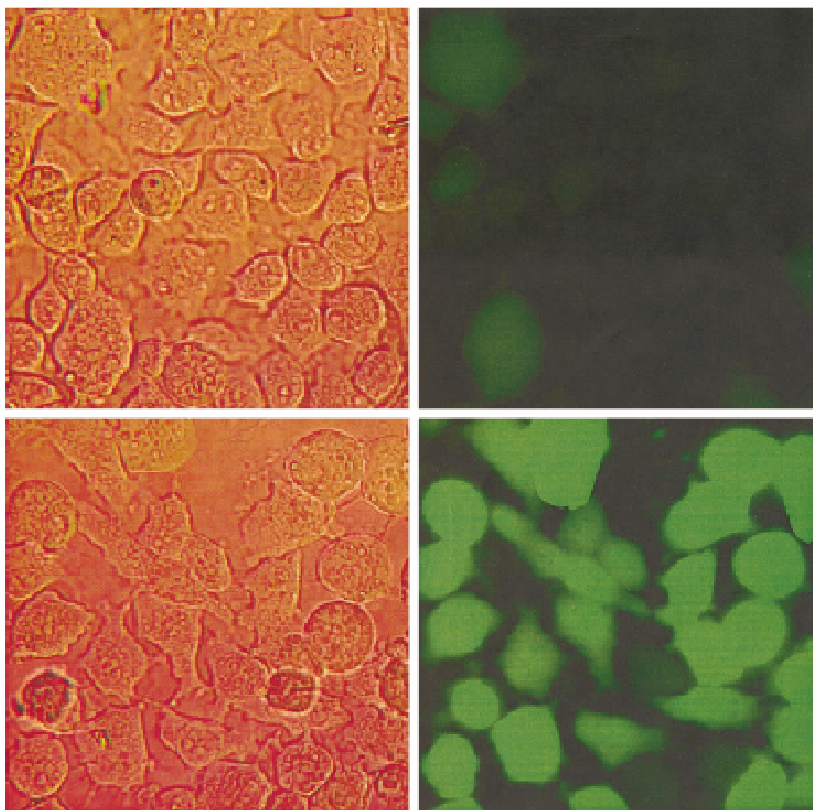
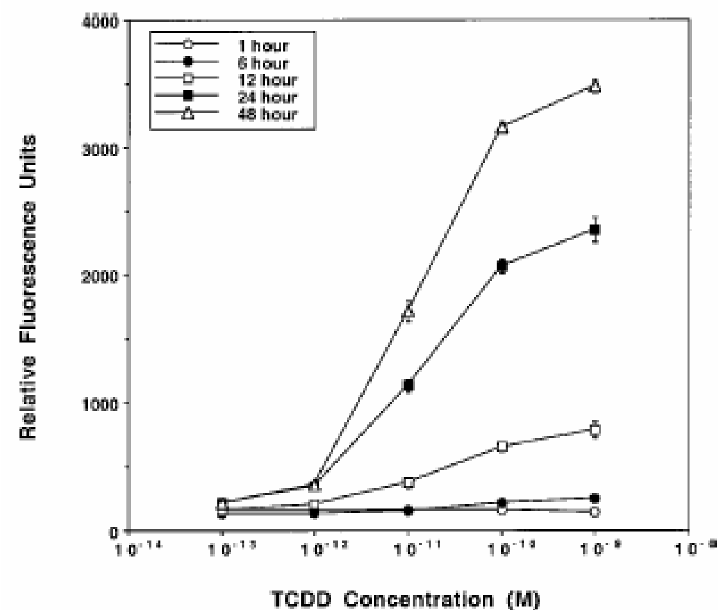
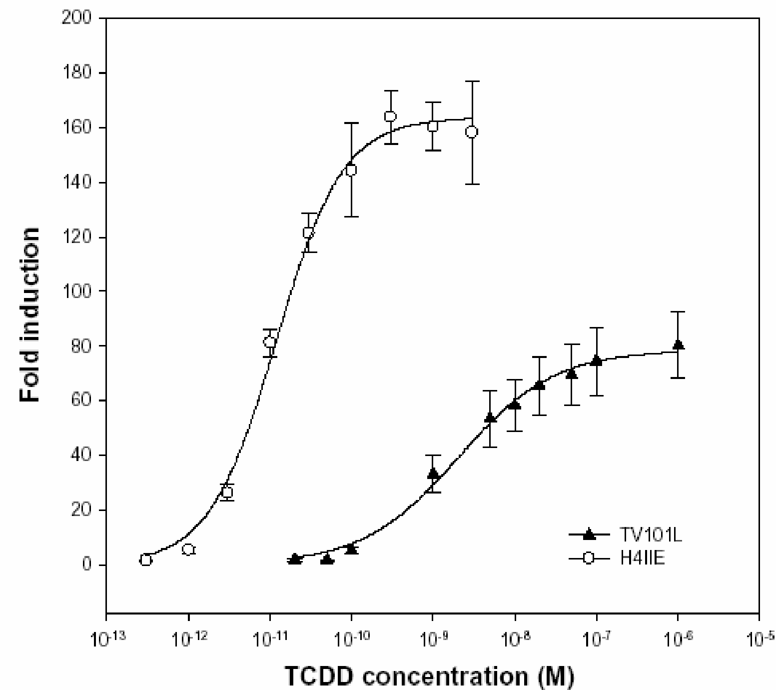


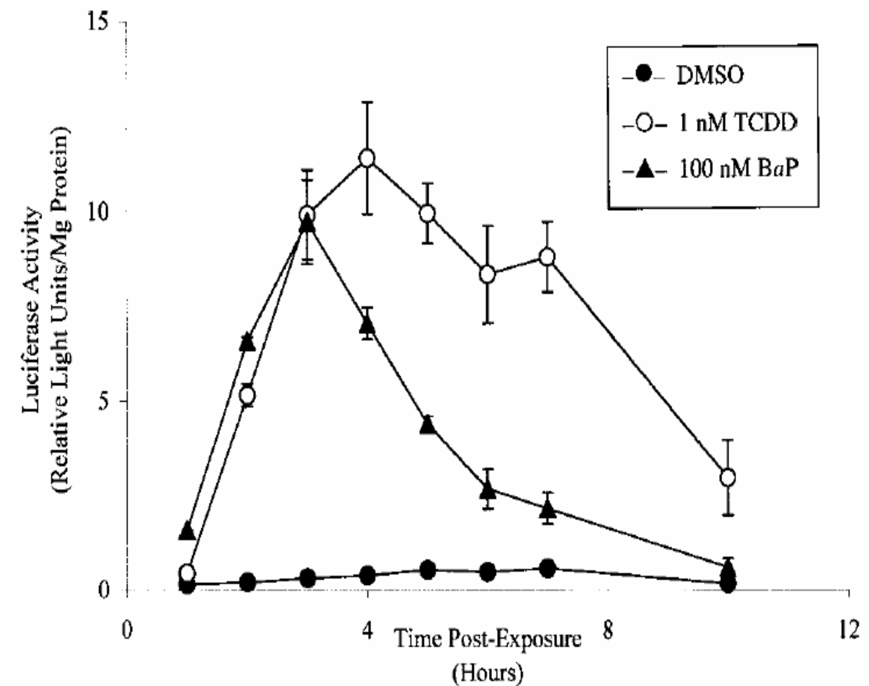
FIG. 3. Microscopy of EGFP1.1 cells incubated in the presence of DMSO or TCDD. H1G1.1c3 cells were grown on 25-mm cover slips until 80% confluent and then treated with DMSO or 10^{-9} M TCDD for 48 h at 33°C. Cells were visualized under differential interference contrast microscopy, or under fluorescence microscopy as described in Materials and Methods.



Luciferase reporter

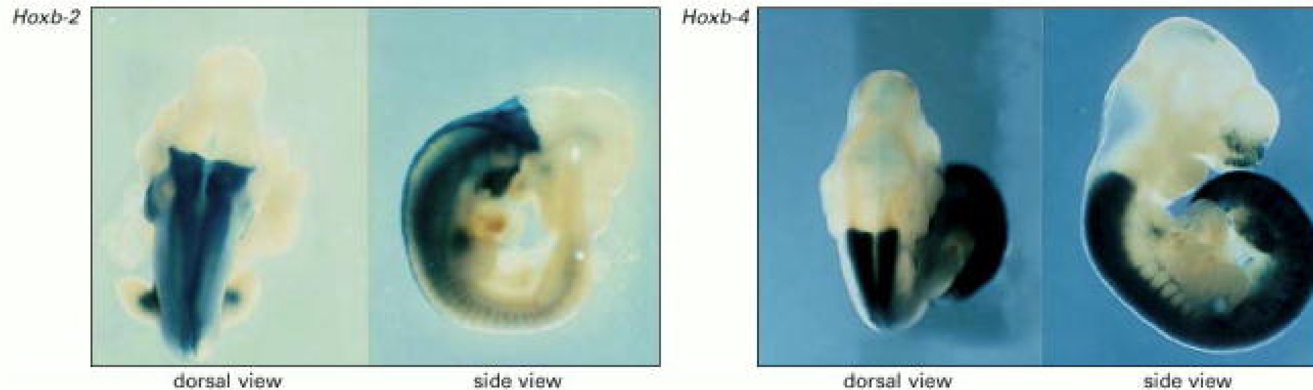
- možnost sledovat dynamiku odpovědi





β-Galactosidase reporter

- možnost využití k in vivo experimentům;

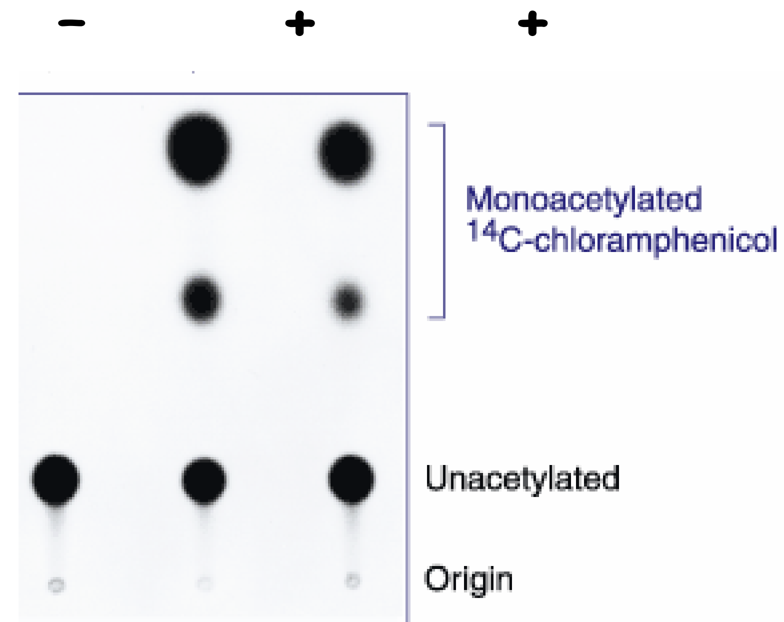


Expression domains of Hox genes in a mouse. The photographs show whole embryos displaying the expression domains of two genes of the HoxB complex (*blue* stain). These domains can be revealed by *in situ* hybridization or, as in these examples, by constructing transgenic mice containing the control sequence of a Hox gene coupled to a *LacZ* reporter gene, whose product is detected histochemically. Each gene is expressed in a long expanse of tissue with a sharply defined anterior limit. The earlier the position of the gene in its chromosomal complex, the more anterior the anatomical limit of its expression. Thus, with minor exceptions, the anatomical domains of the successive genes form a nested set, ordered according to the ordering of the genes in the chromosomal complex.

CAT reporter

- nevýhodou je radioaktivní detekce; často pracná

Thin layer chromatography



- funkce jednotlivých domén;
- posttranslační modifikace (fosforylace, acetylace, glykosylace);
- regulace odbourávání proteinu (ubikvitinace; proteazomální degradace);
- manipulace s proteinem (overexprese; dominant negative constructs, antisense oligonucleotides, siRNA).