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(54) **METHOD FOR TESTING HORMONAL EFFECTS OF SUBSTANCES**

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(57) **ABSTRACT**

The method for testing of substances for hormonal effects, especially for androgenic or anti-androgenic effects, includes exposing cells transfected with two vectors to the substances, wherein one vector contains a DNA, which codes for a nuclear receptor protein, or a fragment thereof, especially a human nuclear receptor protein, or a fragment thereof, and the other vector contains a DNA, which codes for the HSRNAAM co-modulator, or a fragment thereof; and measuring transcription activity, which the nuclear receptor protein, or its fragment, activates or releases in the presence of the HSRNAAM co-modulator, or its fragment, and/or measuring the influence of the substance on the interaction between the nuclear receptor protein, or its fragment, and the HSRNAAM co-modulator, or its fragment, by protein-protein interaction or by protein-protein-DNA interaction. Also a method for determining interference in the co-modulation mechanism between androgen receptor protein and HSRNAAM co-modulator is described.

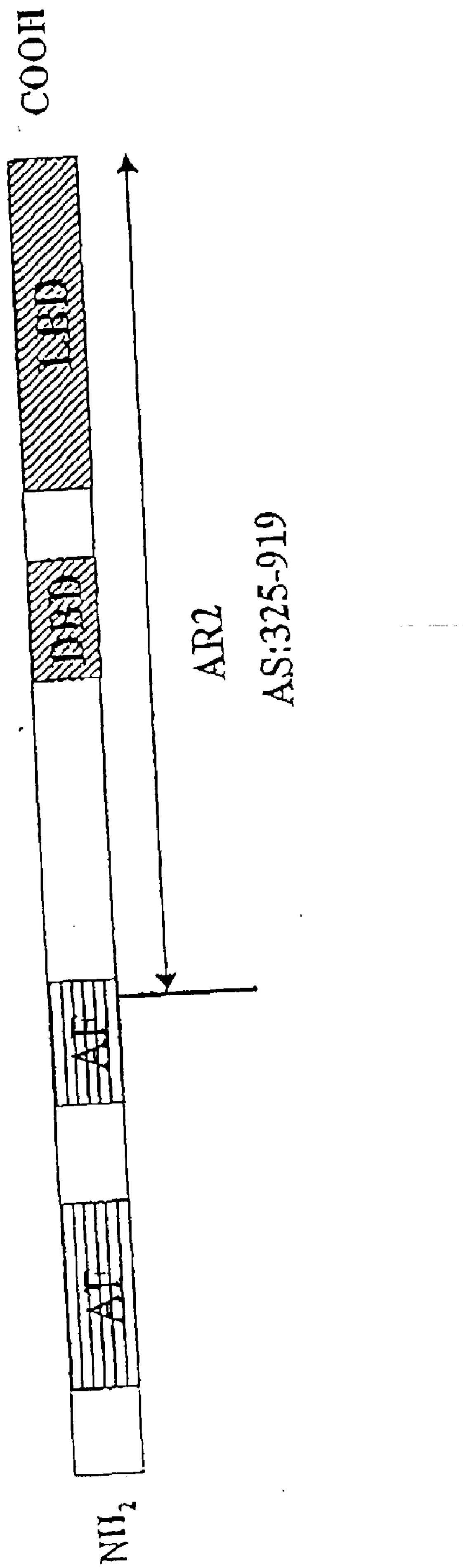


Fig. 1

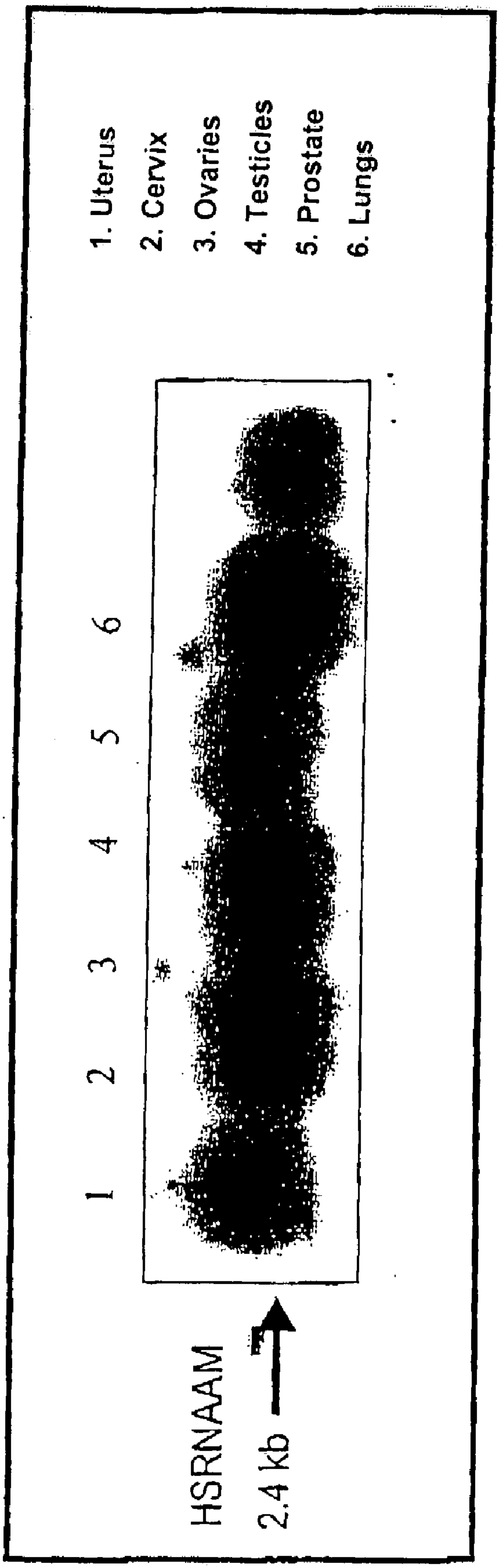

1. Uterus
2. Cervix
3. Ovaries
4. Testicles
5. Prostate
6. Lungs
1 2 3 4 5 6
HSRNAAM
2.4 kb

Fig. 2

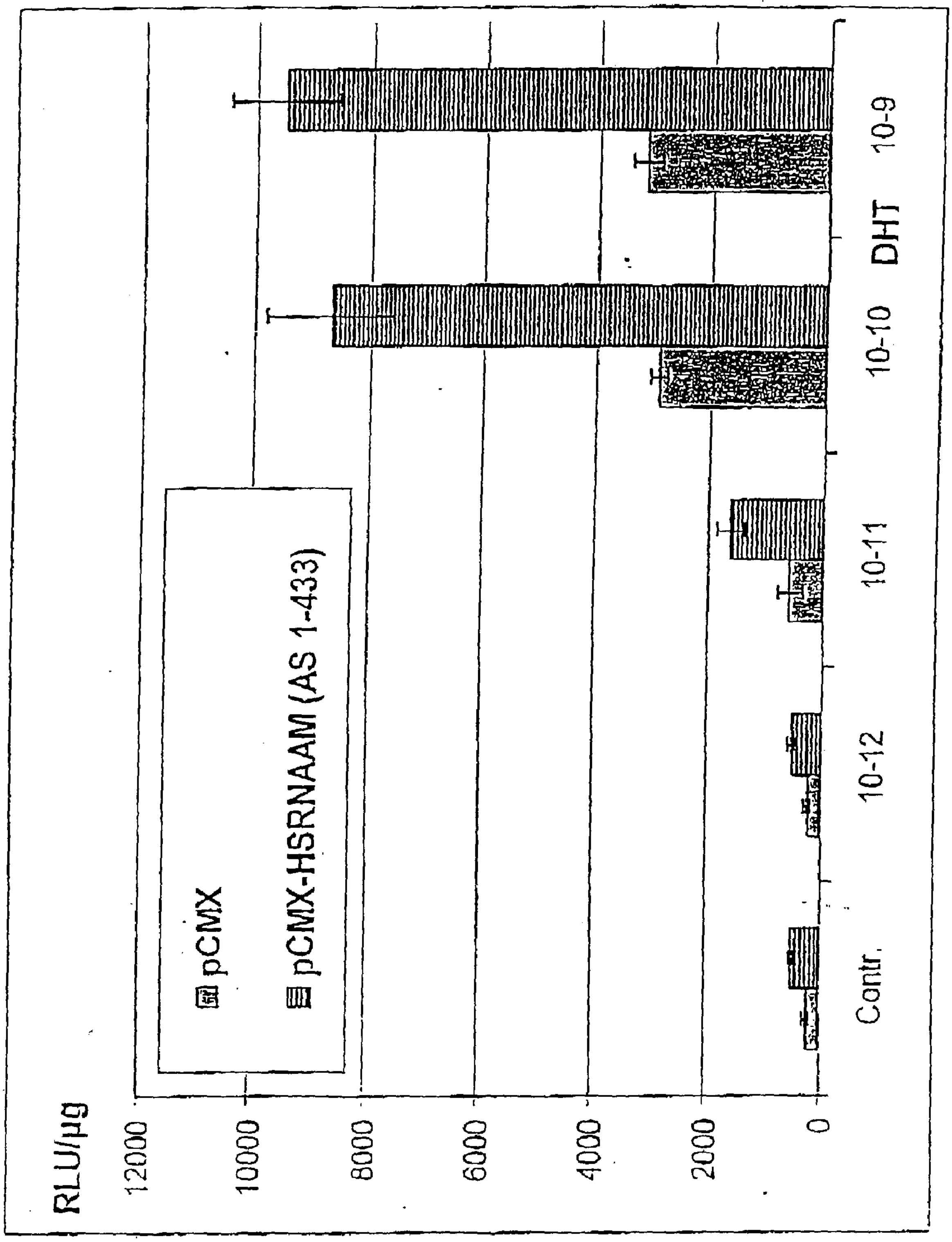
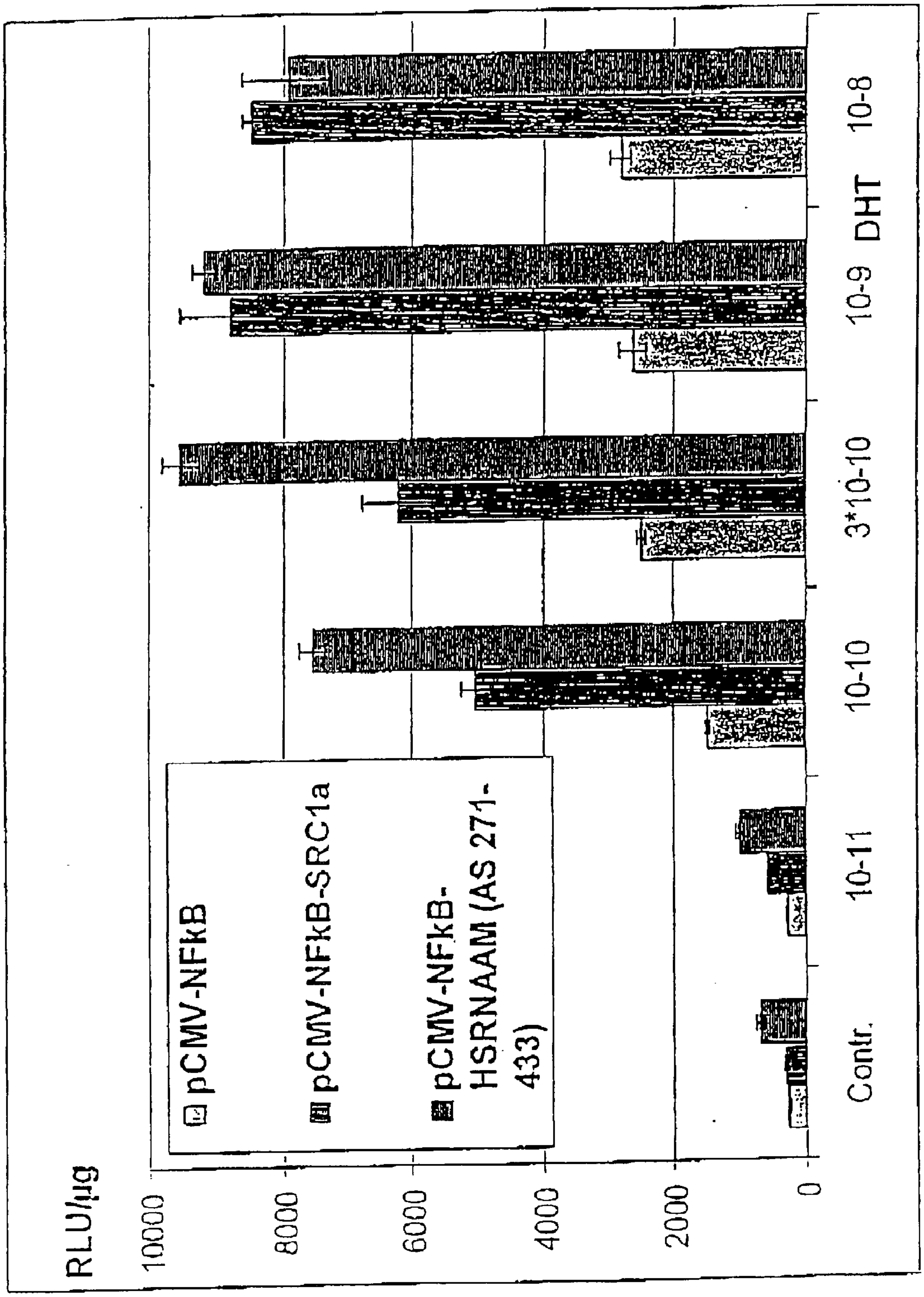

RLU/µg
12000
10000
8000
6000
4000
2000
0
pCMX
pCMX-HSRNAAM (AS 1-433)
Contr.
10-12
10-11
10-10
DHT
10-9

Fig. 3



RLU/µg
10000
8000
6000
4000
2000
0
pCMV-NFkB
pCMV-NFkB-SRC1a
pCMV-NFkB-HSRNAAM (AS 271-433)
Contr.
10-11
10-10
3*10-10
10-9
10-8
DHT

Fig. 4

METHOD FOR TESTING HORMONAL EFFECTS OF SUBSTANCES

BACKGROUND OF THE INVENTION

[0001] The present invention relates to a method of testing the hormonal effect of substances, and, particularly, to a method of determining interference or disturbance in the co-modulation mechanism between androgen receptor proteins and the coactivator arginine methyltransferase (HSRNAAM).

[0002] During evaluation of substances for possible pharmaceutical use it is generally common to test these substances for contingent hormonal action, especially for possible androgenic or anti-androgenic activity. Knowledge of the hormonal effects, especially of androgenic or anti-androgenic effects, of these substances is important in many cases in administration of pharmacologically active substances, since they can bring about undesirable side effects in patients. To test the hormonal action of the various substances, the ability of the substances to bind to hormonal receptors and activate their transcription activity is especially measured.

[0003] Knowledge of the hormonal effects of substances is of interest not only for potential pharmaceutical, but also for non-pharmaceutical, substances, since it is assumed that many substances present in the surroundings can have androgenic or anti-androgenic and/or estrogenic or anti-estrogenic activity. It is possible that some of these substances produce undesirable deleterious effects.

[0004] There is also a considerable need for a method and for a suitable means for performing the method, with which an answer regarding the hormonal effects of substances can be obtained in a reliable, sensitive, simple, economical and rapid manner. The currently known methods are not sufficient.

SUMMARY OF THE INVENTION

[0005] It is therefore an object of the present invention to provide a method and a suitable means for obtaining information regarding hormonal effects of substances to be tested in a reliable, sensitive, simple, economical and rapid manner.

[0006] According to the invention this object is attained in a surprising manner by a method for testing of a substance for hormonal effects, especially for androgenic or anti-androgenic effects, comprising the steps of:

[0007] a) exposing cells transfected with two vectors to the substance, wherein one vector contains a DNA, which codes for a nuclear receptor protein or a fragment thereof, especially a human nuclear receptor protein or a fragment thereof, and the other vector contains a DNA, which codes for the HSRNAAM comodulator or a fragment thereof; and

[0008] b) measuring transcription activity, which the nuclear receptor protein, or its fragment, activates or releases in the presence of the HSRNAAM comodulator, or its fragment, and/or the influence of the substance on the interaction between the nuclear receptor protein, or its fragment, and HSRNAAM comodulator, or its fragment, by protein-protein interaction or protein-protein-DNA interaction.

[0009] It was surprisingly found that whether or not substances, which, for example, can be environmentally relevant or of pharmacological interest, have a hormonal effect, especially an androgenic or anti-androgenic effect, can be determined with the method according to the invention in a reliable, sensitive, rapid, simple and economical manner.

[0010] In the method according to the invention cells transformed with a vector are used. The vector has DNA, which codes for a nuclear receptor protein or a fragment thereof.

[0011] The super-family of the nuclear receptors (NRs), to which more than 50 different proteins belong, is a group of related transcription factors, which control the transcription of respective target genes like reactions at specific ligands, e.g. hormones. The families can be divided into subfamilies according to certain characteristics, such as e.g. dimerization status, the type of ligands or the structure of the DNA reaction elements (Beato, et al, Human Reproduction. Update, 6, pp. 225 to 236 (2000)). The conforming or corresponding structure of the functional domain (designated A to F) is a characteristic feature of the NRs. It has a strongly variable, only weakly preservative, N-terminal region with autonomic constitutive activating function (AF-1) a strongly conservative DNA binding domain (DBD), which is responsible for detection of special DNA reaction elements and comprises two zinc finger motifs. It has a variable hinged domain and conservative multifunctional C-terminal ligand-binding domain (LDB) with dimerization and ligand-dependent transactivation function (AF-2). Following that there is a region at the furthest terminal carbon, whose function is not known and absent in some receptors, such as PR (progesterone receptor), PPAR (peroxisome proliferator-activated receptor) and RXR (retinoid-X-receptor) (Mangelsdorf & Evans, Cell, 83, pp. 841-850 (1995); Robyr, et al, Mol. Endocrinol., 14, pp. 329 to 347 (2000)). For a few NRs (e.g. Androgen-receptors (AR)) it is known that the N-terminal region is in a position to interact with the C-terminal region (Brinkmann, et al, J. Steroid Biochem. And Mol. Biol., 69, pp. 307-313 (1999)). Steroid hormone receptors, such as e.g. estrogen (ER), progesterone (PR), glucocorticoid (GR), mineral corticoid (MR) and androgen receptors (AR) bind steroidal ligands, which are derived from pregnenolone, such as progestin, the estrogens, the glucocorticoids and the mineral corticoids, as well as the androgens, bind steroid ligands. The ligand binding activates the receptors and controls expression of the suitable target genes.

[0012] As previously explained in step a) of the method according to the invention cells are used which contain a vector which contains DNA coding for the co-modulator HSRNAAM or a fragment thereof.

[0013] The so-called co-modulators are classes of proteins, which act as bridging molecules between the transcription initiation complex and the NRs in activation (co-activation) and/or repression (co-repression) of gene transcription (McKenna, et al, Endocr. Rev., 20, pp. 321 to 347 (1999)). A co-activator must be able to amplify or magnify the receptor function and to directly integrate with the activated domains of NRs in the presence of an agonist. It must also interact with the basal transcription apparatus and finally it may not amplify the basal transcription activation by itself. Most co-modulators interact with the help of one or more LXXLL-motif(s) (NR boxes) with the AF-2 domain of NRs. However some co-modulators were described which interact with other NR regions (Ding, et al, Mol. Endocrinol., 12, pp. 302 to 313 (1998)). Chao Qui, et al, describe the protein PRMT2 (protein arginine methyltransferase 2) as co-activator for ERα (JBC Papers in Press, Manuscript M201053200, May 30, 2002).

[0014] In the method according to the invention the co-modulator designated with arginine methyl transferase (HSRNAAM) or especially the fragment of HSRNAAM comprising amino acids 271 to 433 is used. The c-DNA sequence has been described (Genbank X99209) and code for 433 amino acids (Scott, et al, Genomics, 48, pp. 330 to 349 (1998)). The DH5 alpha *E. coli* clones, which contain plasmids coding for the amino acids 1 to 433 and/or 271 to 433 of HSRNAAM, were deposited in the German Collection for Microorganisms and Cell Cultures on Jun. 5, 2002 under DSM 15041 and DSM 15402.

[0015] The method according to the invention can be performed using these proteins in an especially reliable, sensitive, simple, economical and rapid manner. Furthermore the HSRNAAM fragments, especially the fragments having amino acids 271 to 433 of HSRNAAM, have the advantage that they are easily manipulated and cloned, however they still have the functional properties of HSRNAAM.

[0016] HSRNAAM is a co-activator for human androgen receptors and other nuclear receptors, which amplifies the interaction between an androgen and the receptor. The sequence of HSRNAAM is already described in Genbank X99209; generally no interaction with nuclear receptors, especially the androgen receptor, is described there. The invention is based on the surprising knowledge or understanding that nuclear receptors, especially the AR, on the one hand, and HSRNAAM, on the other hand, interact and the AR-mediated transactivation is magnified or augmented. HSRNAAM is a protein, which functions as co-mediator, since it amplifies or represses the transcription effect after binding of steroids to the nuclear receptors and promotes binding and activation of nuclear receptors to molecules, to which no hormonal activation was attributed formerly.

[0017] HSRNAAM represents a co-activator for the androgen receptor and other nuclear receptors, such as estrogen receptor α, estrogen receptor β, progesterone receptor A, progesterone receptor B, glucocorticoid receptor, mineral corticoid receptor, thyroid gland hormone receptor, Vitamin-D receptor, peroxisome proliferator-activater receptor, retinic acid receptor, retinoid X receptor and orphan receptors. These receptors are preferred for use in the present invention, since the advantages of the method according to the invention are obtained in an especially good manner with them.

[0018] Vectors, which code for fragments of the preceding or above-mentioned proteins, can also be used in the method according to the invention. The expression "fragments" should be understood in connection with aforementioned proteins, which have an amino acid or several amino acids less than the full length proteins and still have the functioning properties of a nuclear receptor or a co-modulator.

[0019] As already described above, in step a) of the method according to the present invention cells that are transfected with two vectors, which contain DNA coding for special proteins, are used. These cells are thus in a position to express both different proteins.

[0020] Preferably the cells are from established cell lines and/or eukaryotic cells, especially prostate cells, nerve cells, glia cells, fibroblasts, blood cells, osteoblasts, osteoclasts, hepatocytes, epithelial cells or muscle cells. The method according to the invention can be performed rapidly and economically with the established cell lines. Especially advantageous results can be obtained using the eukaryotic cells, especially the above-described eukaryotic cells.

[0021] In a preferred embodiment of the method according to the invention eukaryotic expression vectors are used, e.g. pCMX, pCMV or pSG5. The method according to the invention can be performed in an especially advantageous and rapid manner and especially outstanding results can be obtained using these vectors, especially in combination with the above-mentioned stable cell lines and/or eukaryotic cells.

[0022] Methods for insertion of the DNA coding the preceding proteins in vectors, for introducing the vectors into cells and for culturing the cells so obtained under suitable culture conditions so that these proteins can be expressed, and materials required for those purposes, are known to those skilled in the art.

[0023] According to step b) of the method according to the invention the transcription activity is measured, which the nuclear receptor protein or its fragment produces in the presence of the co-modulator or its fragment. This can occur, for example, by detection of a reporter gene.

[0024] Reporter genes are genes or gene fragments, which are coupled with other genes or regulator sequences, in order to make the activity of these sequences detectable. Reporter genes produce gene products, which are as easily detectable as possible, for example photometrically by color reaction. Frequently used reporter genes are the genes for β-galactosidase, the gene for alkaline phosphatase, the gene for chloramphenicol-acetyl transferase, the gene for catechol dioxygenase, the gene for the "green fluorescent protein" and different Luciferase genes, which induce the cells to product light.

[0025] These reporter genes can likewise be introduced into the cells with vectors, especially eukaryotic expression vectors. For example a vector, which contains DNA coding for a reporter gene, is the MMTV Luciferase vector, which is used for measuring the androgenic activity of substances.

[0026] Substances with a hormonal effect, especially with an androgenic/antiandrogenic effect, are then detectable by an elevated or reduced activity of the reporter gene.

[0027] The measurement of the influence of the test substance on the interaction between the receptor or its fragment and the co-modulator or its fragment can also occur by determination of the protein-protein interaction. For example, this can take place by twin hybrid systems, immune precipitation, GST pull-down assays, FRET analysis and ABCD assays and determination of protein-protein DNA interaction, for example by gel retardation assays.

[0028] It has been found further that HSRNAAM can be used as a very good indicator of androgenic-conditioned maladies or illnesses. Relevant androgenic-conditioning illnesses or maladies, such as prostate cancer, erectile dysfunction, infertility, grain or glaze formation, acne or hypogonadism and androgen resistant syndromes, such as testicular feminization, are based on defects in or interference with the co-modulation mechanism between AR and HSRNAAM. In patients with these types of illnesses the possibility exists for measurement of relative concentrations of AR and HSRNAAM outside the body. This is possible by use of quantitative methods for measuring relative amounts of both molecules in respective patients, in which for example antibodies can be used both against AR and also against HSRNAAM or nucleic acid probes can be used against their mRNA. There are several methods for measuring these comparative processes, which are known to one skilled in the art. One skilled in the art also knows suitable materials and apparatus for use in these methods. These methods include radioimmunoassay, ELISA tests, immunodyes, RT-PCR, Western Blot or Northern Blot, DNA chip or protein chip. Furthermore it is possible to construct probes for a PCR assay in the usual manner with the help of the HSRNAAM-cDNA. Mutations of the normal DNA sequence are detected in certain patients or transcriptions for Northern Blot Assay and/or a DNA for In situ hybridization assays may be produced with these latter probes.

[0029] The measured ratio of AR to HSRNAAM can be greater or smaller than that required in healthy individuals. The normal value in healthy individuals can be determined in a simple manner, for example by measuring the ratio of AR to HSRNAAM in number of healthy test subjects. By comparison of this normal value with that measured in a patient to be tested it can be established whether or not the value in the patient is greater or less than the normal value.

[0030] The concentration of HSRNAAM and/or AR can be different in different tissues. For example the concentration of HSRNAAM in the antechamber of the heart, the marrow, the thymus and uterus is very large, while in contrast the concentration in the liver, lungs and prostate can be comparatively smaller. The different concentrations in the different tissues must be considered during the testing. That means that the test value and the normal or standard value compared should be from the same type of tissue.

[0031] Another possibility for determination of defects in the co-modulation mechanism between AR and HSRNAAM can be based on only measuring only the concentration of HSRNAAM, while assuming that the AR concentration is at least approximately constant. If a less than normal HSRNAAM concentration is measured, that means that the ratio of AR to HSRNAAM has shifted, which suggests interference with the co-modulation mechanism.

[0032] It is also possible to determine changes in the HSRNAAM expression and thus in the ratio of it to AR with an HSRNAAM specific probe. These changes can be involved in starting different illnesses or as consequence of them.

[0033] This surprising knowledge that, for example, an androgen resistant syndrome can be based on interference or disturbance of the equilibrium between AR and HSRNAAM prevalent in the target cells rests on the finding and characterization of HSRNAAM as co-modulator obtained from the measurements of the AR/HSRNAAM ratios. Too much HSRNAAM can lead to an over-sensitivity of the AR system, so that it reacts to molecules, which normally have no androgenic effect. The reverse leads to the absence or missing function of HSRNAAM to all levels of androgen resistance. The detection of too much HSRNAAM in patients calls for use of means for down-regulation, such as anti-sense or similar medicines, in order to reduce the HSRNAAM titer in certain patients under clinical conditions. This can be achieved by molecules, which are in a position to inhibit the interaction between AR and HSRNAAM. If a patient has too little HSRNAAM, then HSRNAAM-cDNA, -protein or -DNA can be administered to him by different known mechanisms to increase the titer of the active HSRNAAM in this way. It is possible also to increase the concentration or the activity of the HSRNAAM by small molecule drugs or by stimulation of the self-synthesis with the aid of specific HSRNAAM promoter proteins.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

[0034] The objects, features and advantages of the invention will now be illustrated in more detail with the aid of the following description of the preferred embodiments, with reference to the accompanying figures in which:

[0035] **FIG. 1** is a schematic diagram of the androgen receptor showing the androgen receptor domain (AR2) from amino acid 325 to 919, which is able to interact with HSRNAAM in the presence of androgens;

[0036] **FIG. 2** is an illustration showing the distribution of HSRNAAM in various different human tissues;

[0037] **FIG. 3** is a graphical illustration showing the action of HSRNAAM (AS 1-433) as co-modulator with the androgen receptor in PC3-ARwt cells; and

[0038] **FIG. 4** is a graphical illustration showing the interaction of an HSRNAAM fragment (AS 271-433) or SRC-1a with the androgen receptor in PC3-ARwt cells.

[0039] The following examples serve to illustrate the claimed invention without further limiting it.

EXAMPLES

Example 1

[0040] A screening by a conventional two hybrid yeast system in the presence of androgen 10^{-6} M DHT is performed using a cDNA library from fetal liver (Clontech MATCHMAKER) and a human AR fragment, which codes for the amino acids 325 to 919, as probe. In agreement with the recommendations of the manufacturer (Clontech) the number of screened clones amounted to 2×10^{7}. The number of independent clones amounts to 3.5×10^{6} according to the public statements of the manufacturer. From those 300 positive clones were selected and tested with a β-galactosidase assay. Of those latter clones 40 were reported as lacZ-positive clones. The inserts of these clones were amplified with PCR. At least 39 different clones were identified by restriction fragment analysis and sequencing. One of those was a clone with an insert comprising 1110 bp (986 bp to 2096 bp), which coded for a part of the ORF (open reading frame) of HSRNAAM (Genbank access number X99209).

[0041] The fragment comprising 499 bp (986 bp to 1485 bp) of HSRNAAM-cDNA sequence served as probe for human blot. The tissue distribution of the arginine methyl transferase was determined (**FIG. 2**) by a Northern Lights Human Multiple mRNA Blot (Life Technologies). A transcript was found using the HSRNAAM probe.

[0042] **FIG. 2** shows the tissue distribution of HSRNAAM, which was tested by a Northern Blot Analysis in a known manner. Poly-A^+-RNA isolated from different human tissues, which was normalized to β-aktin, was separated with a formaldehyde-containing agarose gel, blotted on a NYLON® membrane and hybridized with a normalized HSRNAAM-cDNA fragment (986 to 1485 bp). A transcript was found in different human tissues (**FIG. 2**).

[0043] The HSRNAAM-cDNA fragment from 986 bp to 1485 bp (AS 271-433) amplified by the PCR and specific primer from the yeast vector was cloned with the endonucleases EcoRI and XhoI in the usual way in the vector pCMV-NFκB and the HSRNAAM-cDNA fragment from 177 bp to 1485 bp (AS 1-433) amplified by specific primer from human universal cDNA (Clontech) was cloned with the endonucleases EcoRI and XhoI in the usual way in the vector pCMX and with MMTV luciferase in PC3-ARwt cells, which express AR, similarly transfixed.

[0044] As shown in **FIG. 3** the transient transfection of HSRNAAM-cDNA (AS 1-433) in PC3-Arwt cells leads to a strong co-modulation of the AR signal activity and the arginine methyl transferase acts as co-activator for the nuclear receptor. Further the transient transfection of the HSRBAAM-cDNA fragment (AS 271-433) fused in frame with the transactivated domain from NFκB in PC3-Arwt cells leads to a strong co-activation of the AR signal activity (**FIG. 4**), which indicates an interaction between the arginine methyl transferase and the nuclear receptor or other co-modulators. Those were transfixed with 1.0 μg MMTV luciferase plasmid and with 0.5 μg of pCMX-HSRNAAM construct (AS 1-433) and/or with 0.35 μg pCMV-NFκB as negative control or with 0.5 μg pCMV-NFκB-SRC1a as positive control in respective cavities having 2×10^5 cells per cavity in a cell culture dish. The transfixed cells were treated 24 hours with dihydroxytestosterone (DHT) in the stated concentrations and harvested after another 24 hours, before measuring the activity of the reporter gene (Luciferase). Additionally the entire cell protein amounts were determined for normalization. Two experiments with three measurements each were performed for each transfection initiation and substance concentration. The error variation was reported as SD. The activity is given in relative units.

[0045] The disclosure in German Patent Application 102 26 675.1 of Jun. 12, 2002 is incorporated here by reference. This German Patent Application describes the invention described hereinabove and claimed in the claims appended hereinbelow and provides the basis for a claim of priority for the instant invention under 35 U.S.C. 119.

[0046] While the invention has been illustrated and described as embodied in a method for testing for hormonal effects of substances, it is not intended to be limited to the details shown, since various modifications and changes may be made without departing in any way from the spirit of the present invention.

[0047] Without further analysis, the foregoing will so fully reveal the gist of the present invention that others can, by applying current knowledge, readily adapt it for various applications without omitting features that, from the standpoint of prior art, fairly constitute essential characteristics of the generic or specific aspects of this invention.

[0048] What is claimed is new and is set forth in the following appended claims.

We claim:

1. A method for testing a substance for hormonal effects, said method comprising the steps of:

a) exposing cells transfected with two vectors to the substance, wherein one of the two vectors contains a DNA, which codes for a nuclear receptor protein, or a fragment of said nuclear receptor protein, and another of the two vectors contains a DNA, which codes for an HSRNAAM co-modulator, or a fragment of said HSRNAAM co-modulator; and

b) measuring transcription activity, which said nuclear receptor protein, or said fragment of said nuclear receptor protein, activates or releases in the presence of the HSRNAAM co-modulator or said fragment of said HSRNAAM co-modulator, and/or measuring an effect or influence of the substance on an interaction between said nuclear receptor protein, or said fragment of said nuclear receptor protein, and the HSRNAAM co-modulator, or said fragment of said HSRNAAM co-modulator, by protein-protein interaction or by protein-protein-DNA interaction.

2. The method as defined in claim 1, wherein said hormonal effects are androgenic or anti-androgenic effects.

3. The method as defined in claim 1, wherein said nuclear receptor protein is a human nuclear receptor protein, or said fragment of said nuclear receptor protein is a fragment of said human nuclear receptor protein.

4. The method as defined in claim 1, wherein said fragment of said HSRNAAM co-modulator has amine acids 271 to 433.

5. The method as defined in claim 1, wherein said nuclear receptor protein is selected from the group consisting of androgen receptor, estrogen receptor α, estrogen receptor β, progesterone receptor A, progesterone receptor B, glucocorticoid receptor, mineral corticoid receptor, thyroid gland hormone receptor, Vitamin-D receptor, peroxisome proliferator-activer receptor, retinic acid receptor, retinoid X receptor and orphan receptors.

6. The method as defined in claim 1, wherein said cells are selected from established cell lines and/or are eukaryotic cells.

7. The method as defined in claim 6, wherein said eukaryotic cells are selected from the group consisting of prostate cells, nerve cells, glia cells, fibroblast cells, blood cells, osteoblast cells, osteoclast cells, hepatocytes, epithelial cells and muscle cells.

8. The method as defined in claim 1, wherein said vector is a eukaryotic expression vector.

9. A method for determining interference in a co-modulation mechanism between androgen receptor protein and HSRNAAM co-modulator, said method comprising measuring a concentration of HSRNAAM co-modulator, or a fragment thereof, and/or measuring a concentration of androgen receptor protein, or a fragment thereof.

10. The method as defined in claim 9, wherein said measuring of said concentration and/or said concentrations takes place by radioimmunoassay, ELISA test, immunodyeing, RT-PCR, Western Blot or Northern Blot.

* * * * *

专利名称(译)	测试物质的激素作用的方法		
公开(公告)号	US20030232376A1	公开(公告)日	2003-12-18
申请号	US10/456269	申请日	2003-06-06
[标]申请(专利权)人(译)	WOLF西格蒙德 OBENDORF MAIK MEYER RENE 施罗德JENS		
申请(专利权)人(译)	WOLF西格蒙德 OBENDORF MAIK MEYER RENE 施罗德JENS		
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发明人	WOLF, SIEGMUND OBENDORF, MAIK MEYER, RENE SCHROEDER, JENS		
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摘要(译)

用于测试激素作用物质的方法，特别是用于雄激素或抗雄激素作用的物质，包括将用两种载体转染的细胞暴露于物质，其中一种载体含有编码核受体蛋白的DNA，或其片段，特别是人核受体蛋白或其片段，另一载体含有编码HSRNAAM共调节子的DNA或其片段;测量转录活性，核受体蛋白或其片段在HSRNAAM共调节剂或其片段存在下激活或释放，和/或测量物质对核受体蛋白之间相互作用的影响，或其片段，以及HSRNAAM共调节剂或其片段，通过蛋白质 - 蛋白质相互作用或通过蛋白质 - 蛋白质-DNA相互作用。还描述了用于确定雄激素受体蛋白和HSRNAAM共调节剂之间的共调节机制中的干扰的方法。

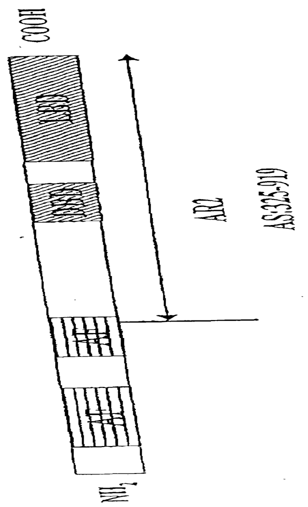

Fig. 1