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发展中的mRNA差别显示技术

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摘要:

随着PCR技术的出现(1985)，在分子生物学界又相继出现了两个很有影响的新技术——RAPD技术(1990)和mRNA差示法(1992)，前者用于分子标记，后者用于基因分离。

mRNA差示法的生物学基础是基因的差别表达，既:单个细胞中表达的基因仅占基因总数的15%。这种基因的差别表达决定了生命的所有过程，如:发育和分化、对逆境的反应、细胞分裂、老化等。图一给出了该方法最初的技术路线。提取要比较的两种或两种以上样品的mRNAs，分别逆转录成cDNAs，经过PCR扩增后，直接进行测序胶电泳即可识别有差别的mRNA。其中、关键的是PCR扩增时两个引物的设计。3'端引物Oligo(dT)MN很容易与具有N'M'-poly(A)-3'末端的大多数mRNA结合，进行cDNA的逆转录合成。M、N提供锚定位点，防止3'端引物在 poly(A) 序列不同位置上的随机结合。5'端为10个碱基的随机引物。这个经验上的碱基数值较理论的6-7个碱基(表一)更能满足测序胶电泳要求的条件:分子大小在500bp左右，每条泳道上条带数在100条左右。

该方法近年来又有如下改进: 一、PCR退火温度由 42°C改为 40°C，可在保证特异性的同时，增加泳道上的条带数。cDNA合成的底物改用总RNA，可避免poly(dT)柱纯化mRNA时的污染，造成电泳抹带(smear)现象。二、第二代引物3'端的M位(3'端倒数第二位)碱基改用4 个简并碱基，减少了引物的组合数，提高该法的效率和经济性。第三代引物在两种引物的 5'端加*Hind* III酶切位点，3'端引物改为单随机碱基，既: 5'-AAGCT11N-3'; 5'端引物为 5'-AAGCTT+(AP)7。大大提高了克隆效率和克隆cDNA的可操作性。三、可能由于10个碱基随机引物的特异性还不够，泳道上出现的cDNA条带数多得超过了可分辨的程度，造成了假阳性现象。有人尝试用不变性凝胶代替变性凝胶、或用Northern亲和层析加以解决。收效不大。也许，还是需要在引物设计上下功夫。四、经比较，^{33}P 的半衰期和放射性强度介于 ^{32}P和^{35}S之间，价格最贵，但效果最好。

通过对正常细胞与病变细胞、处理与未处理细胞、不同生理状态和不同发育时期的细胞，以及抗逆性强弱所作的mRNA差别显示分析，在动植物生理、病理以及抗性研究中取得了大量成果。但是，由于该技术要求底物有poly(A)尾，只能限于真核生物核基因组差异表达的研究。而且，所选择的研究材料除要求目的基因有差异外，其它基因差异要尽可能小。GenHunter 公司已推出了方便的专用试剂盒，但使用的是与人有害的、需要较长曝光时间的放射性同位素，这些都是需要进一步改进的，还包括向技术的自动化改进。

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Progress in mRNA Differential Display[1]

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ABSTRACT: The mRNA Differential Display is a new molecular biological strategy for detecting and characterizing altered gene expression in eukaryotic cells which was developed in 1992. Because of its simplicity, sensitivity and reproducibility, this method should find wide-ranging underpaid application developmental and molecular biology. Therecent successful applications of this method to gene hunting and the technological improvement promise great potential of mRNA Differential Display.

Key Terms: mRNA Differential Display

Since the establishment of Polymerase Chain Reaction (PCR)in 1985, molecular biological approaches were facilitated greatly and developed quickly, many PCR-related techniques have appeared. In 1990, two groups of American scientists created the technique of Randomly Amplified Polymorphic DNA (RAPD) for polymorphism study of amplified band pattern using arbitrary primers, it develops PCR methods from only being used on amplifying an known sequence to be an important technique of molecular marking. Two years later, Peng Liang and Authur Pardee developed a new experimental strategy——mRNA Differential Display on the basis of RAPD and DNA sequencing gels, it provides a simple and convenient method for isolating unknown genes, studying gene expression and other molecular fields.

1. PRINCIPLE AND METHODS

Higher organisms contain about 10^5 different genes, of which only a small fraction, about 15，000 are expressed in any individual cell. It is the choice of which genes are expressed--"differential expression" that determines all life process--development and differentiation, cell cycle regulation, respondent to stress, aging and programmed cell death. Comparison of gene expression in different cell types provide the underlying information we need to analyses and understand the molec-

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ular basis of life[15] . mRNA Differential Display technique is just a kind of experimental design in accordance directly with differential expressed mRNAs to study altered expressed genes, identify and clone these genes. So the key element of this technique is to define mRNAs and to visualize, identify, isolate its difference.

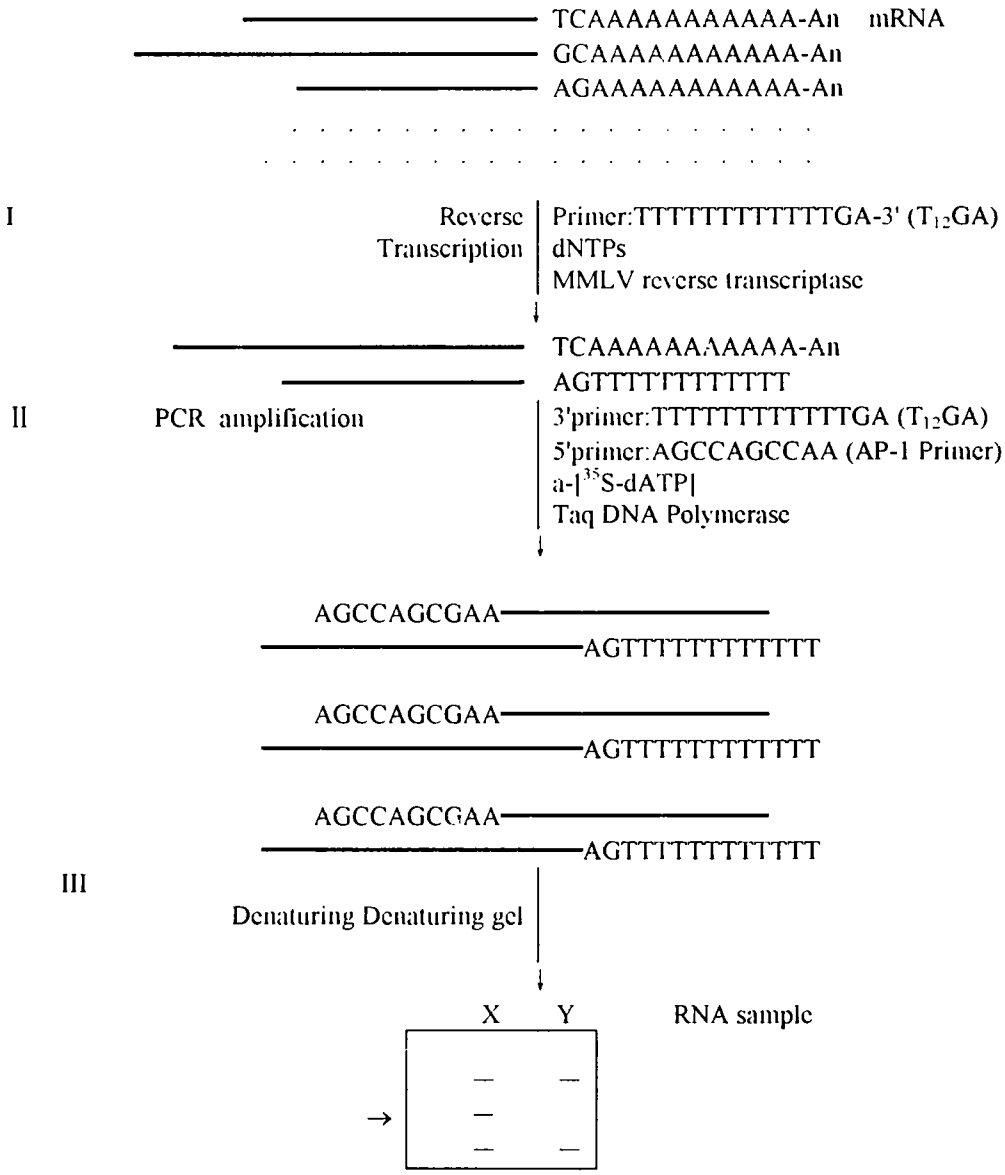

Fig. 1
Primary experimental procedure of mRNA Differential Display developed by Peng Liang and Authur Pardee (1992)[15].

The strategy of mRNA Differential Display is to resolve the 3' terminal portions of mRNAs on a DNA sequencing gel. Using a primer designed to bind to 3' boundary of a poly (A) tail for the reverse transcription, followed by PCR amplification with additional upstream arbitrary primers, mRNA subpopulations are visualized by denaturing polyacrylamide electrophoresis (**Fig. 1**).

It can be seen from the above procedure that the key point of this technique is the primer design.

1.1. 3'primer:

Selection of 3' primers takes advantage of the polyadenytlate [poly (A)] tail present on most eukaryotic mRNAs to anchor the primer at the 3' end of the mRNA, plus two additional 3' bases. Because there are 12 different combinations of the last two 3' bases omitting T as the penultromate base, a primer such as --T_{11}MN will recognize one twelfth of the total mRNAs population by probability. The primer permits initiation of reverse transcription of only this mRNA subpopulation. The two arbitrary bases -- M,N could provide anchoring site to prevent 3'primer from annealing randomly with mRNAs.

1.2. 5'primer:

Because an average molecular size of mRNA is 1.2 Kb, any reverse transcribed cDNA species would be amplified by PCR if the annealing position of a second primer existed. For a 5'primer of arbitrary base sequence, annealing positions to cDNAs should be randomly distributed in distance from poly (A) tail. Therefore, the amplified products from various mRNAs will differ in size and would be visualized by autoradiography as a ladder on a sequencing gel. On a sequencing gel, the visualized molecular size is usually within 500 bps, and 100 bands exhibited in each lane will be best suitable for identifying pattern difference. Theoretically, 6-7mers will be a good choice for this need. But Liang and Authur found that arbitrary 10-mers in conjunction with anchored oligo(dT) primer, can amplify more bands than statistics allows, in theory acting like 6-7mers (**Table 1**) .

This suggests the shorter primers may have too low melting temperatures to bind efficiently, could not amplify the theoretical bands. And this result also suggests that 10-mer primer may hybridize to the target mRNA sequence in a degenerate fashion during PCR cycles. This degeneracy is advantageous in revealing an optimal number of mRNA species per lane. In theory, about 10,000 6-7mer sequences are possible. The chance of finding any one such sequence in 500 bases thus is 0.05. Therefore 20 arbitrary 10-mer (priming as 6-7mer) should statistically cover all mRNA sequences upstream of the 12 possible anchored oligo (dT) primer.

Table 1

The theory and experiment number of mRNA displayed with different length of arbitrary primer[9,10]

Length of arbitrary primer(bases)	kilobases per binding site	mRNA displayed (NO.)	
		Theory	Experiment
6	4	150	0
7	16	38	0
8	65	10	0
9	262	2	20-30
10	1049	<1	50-100

2. REFINEMENTS AND IMPROVEMENTS

Although the general strategy of mRNA Differential Display seems to be straight forward, recent reports on refinements in methods suggested that the involved procedures are technically challenging[15]. The inventors and other reseachers have made it a lot of improvements in many ways:

2.1. Optimization of Reaction Conditions.

Liang et al[12,15] primarily adopted 42℃ as the annealing temperature in the PCR amplification. They found 50-100 bands per lane appeared in average. As the followed experiments were carried on, 40℃ as the annealing temperature was proved to be more favourable. When 40℃ was adopted, more bands per lane appeared. As a result, this could enhance the efficiency under the requirement of specificity. For this reason, most of current research adopted 40℃ as the annealing temperature. As to the reaction template, mRNA was the original choice. However, researchers observed that procedure of purifying mRNA will raise poly(dT) contamination which produces smear in sequencing gels. Therefore, Liang et al.(1993)[9,13] suggested total cellular RNA is preferable. Either mRNA or total cellular RNA will produce nearly identical cDNA patterns by a given set, but the latter can avoid the risk of poly(dT) contamination from the mRNA purification procedure.

2.2. Improvements of Primer Design.

Liang et al.[13] demonstrated that the penultimate base from the 3' end of the oligo(dT) MN exhibit considerable degeneracy during priming in the reverse transcription step and it is the last base from the 3' end that provides most of the specificity. So they attempted to use four different degenerate primers $T_{12}MG$, $T_{12}MA$, $T_{12}MT$ and $T_{12}MC$ for the reverse transcription of RNA. This decreased the number of 3' primers and the combination of 3'primer and 5'primer. As a result, the mRNA Differential Display technique with 2nd generation primers got more efficient

and economic. In 1994, Liang et al.[17] designed new primers further. They added a HindIII restriction site (5'- AAGCTT-3') to the 5' ends of both primers and one - arbitrary-base primers were adopted as the 3'primer. Consequently, 3'primers performed 5'- $AAGCT_{11}N$-3' and 5' primers appeared to be 5'-AAGCTT+$(AP)_7$ (arbitrary primer). The 6 bases of the HindIII site at the 5' ends statistically provides an additional 1.5 base match for any arbitrary sequence. This reformation was intended for both easier manipulation of the amplified cDNAs after cloning and more efficient amplification due to the longer primers used. As to the aspect of amplification efficiency, one mRNA was displayed with the probability of 95% after amplification by all sets of 12 kinds of 3'primers and 25 kinds of 5' primers under the first generation primers $[1-(1-1/4^6)^{500\times25}]$. In average, almost all mRNAs will be visualized on the sequencing gels by all sets of 12 kinds of 3'primers and 20 kinds of 5'primers. However, 80 kinds of 5'primers and 3 kinds of 3'primers are not enough to display all mRNAs under the 3rd generation primers.

2.3. Elimination of False Positive.

False positive is the most difficult problem which disturb researchers. Extrinsic factors such as pipetting errors, integrity of RNA samples, contamination of RNA with chromosomal DNA or even the quality of PCR tubes will result in the false positive[17]. This type of false positive may be distinguished by carrying on a PCR amplification without reverse transcription as the controll. But the false positive due to intrinsic factors is not so easy to be eliminated. Liang et al.[12] reported for the first time in 1992 that the differential cDNA in the sequencing gels can not be confirmed by Northern blot analysis. They believed that the false positive was caused by the lower redundancy of certain mRNAs or short cDNA or high AT percentage in some cDNAs. Clark et al.[7] reported that DNA Polymerase (include the Taq Polymerase) possesses a terminal transferase-like activity. It usually catalyzes non-templated addition of a dA base to the 3'termini of blunt- ended DNA. David et al.[3] confirmed that the non-templated catalyzing activity will make same cDNA separate on the sequencing gels according to whether the cDNA adding a dA base or not. It means a band visualized on the sequencing gels is probably composed of different cDNAs and the false positive produced resultly. Moreover, because 10- arbitrary-base 5'primers hybridize as same as 6-arbitrary-base primers and 3' primers are not 2-arbitrary-base primers but 1-arbitrary-base primers for the degeneracy of the penultimate base, the number of valid 5' priming site is only 4^6=4096, and every 3'primer can produce 15,000/4= 3,750 kinds of mRNAs through reverse transcription in theory. For this reason, the probability of different mRNAs having the same arbitrary-primer binding fragment at the same site is considerable. This may be the most serious reason which results in the false positive. In order to eliminate the intrinsic-origin false positive, David et al.[3] adopted native gels instead of denaturing gels for the electrophoresis. They considered that one-dA-difference cDNAs steming from same mRNA will not

separate on the native gels and the false positive can be overcome. They named this method DDRT-PCR after differential display reverse transcription PCR. What is deserved to mention with the DDRT-PCR is that different cDNA appearing at same band on the denaturing gels may separate on the native gels because of their different secondary structure, but both the length and the secondary structure determine cDNA separating pattern and a problem is caused from it: cDNA separating pattern on the native gels will be not as regular as on the denaturing gels. Whether will this irregularity pattern lead to difficult in the differential cDNA recovering? Furthermore, is it probable that the length-different cDNAs appear at the same band due to the influence of their secondary structure? Whether will this probability produce new false positive? All of the question is worth pondering. There is not any other report about the native gels in the differential display technique at present. Li et al.[18] attempted to solve the problem by means of Northern blot to affinity capture cDNA fragments. Fragments of cDNAs which demonstrated differences in mRNA level were recovered from the membrane after hybridization and cloning. Their experiment showed that the cloned fragments were confirmed by repeated Northern analyses. Callard et al. [4] also found that the purified differential cDNA is still contaminated by irrelevant cDNAs which lead to the false positive. In general, false positive arises during the random amplification. The essence of this problem is that the actual number of cDNAs per lane exceeds the number of detectable bands because 10-arbitrary-base primer can not offer enough specificity. Therefore, the best and key solution of this problem may exist in reformation of the primers.

2.4. Radioactive Label.

At present, all mRNA differential display researches use radioactive isotope as a label . The choice of which kind of isotope to use is determined by several factors, including the sensitivity of detection, band resolution, safety and cost [16]. ^{35}S [15], ^{32}P[12,13,18,25], ^{33}P[3,17,22] were popular used at present. Each of them has its distinguishing feature. ^{35}S is a good labeling isotope for its high resolution, long half-life and sensitive of detection. But its drawback is that volatile ^{35}S-labeled decomposition products may form and contaminate the PCR machine. As for ^{32}P, its half-life is short and radioactive energy is strong. The drawbacks for the routine use of ^{32}P are that physical protection is required and the band resolution is low. ^{33}P may be the best choice except its cost. The half-life and radioactive energy of ^{33}P is just between ^{32}P and ^{35}S. It has not the risk of volatile ^{33}P-labeled decomposition products forming. Many experiments proved that the using of 1uCi α -[^{32}P] dATP can get more sensitive and resolution than the using of 5uCi α -[^{35}S] dATP.

The mRNA Differential Display technique has being refined and consummated since the day it was developed. In 1995, Reeves et al [19] and Wang et al[24] improved this technique and made it possible to sequence the differential fragments without

subcloning. This will save a lot of time and cost further. It was also reported by Rohrwild et al[20] that the primers containing inosine were used.

3. APPLICATION

mRNA Differential Display technique developed by Dr. Peng Liang and Dr. Arthur B.Pardee at Harvard Medical School has taken U.S. patent[9]. With the development of the technique for only three years, mRNA Differential Display has been applicated in various fields and made great progress.

3.1. Cancer Research:

The Differential Display technique was applicated by Liang et al[12] to compare mRNAs from normal and tumor-derived human mammary epithelial cells cultured under the same conditions. mRNA fragments corresponding to several apparently differentially expressed were recovered and sequenced. A cDNA fragment seen only in the normal cells was used as a probe to isolate its corresponding cDNA clone from a library. Liang also used this technique to study mutant Ras genes on the basis of their differential expression in rat embryo fibroblasts, and found a downstream target of the Ras signaling pathway, mob-1[14]. Sanger et al[21] used this techniques to clone a possible tumour- suppressor gene, designated a -6 intergrin.

3.2. Growth Factor and Hormone Regulation.

Li et al[18]used the Differential Display technique as a part of ongoing studies to investigate the effect of basic fibroblast growth factor (bFGF) on human endothelial cells, and clone the special cDNA fragment. Using the mRNA Differential Display, Babalola et al[2] studied the effect of growth factors, α -TGF or β -TGF on regulating gene expression in the preimplantation mouse embryo, finding that either α -TGF or β -TGF results in the increased expression of the b subunit of the F-0 ATPase, β - TGF also stimulates the expression of the DNA polymerase, α -TGF treatment results in the increase in expression of a gene homologous to the human HEPG2 cDNA, as well as results in a decrease in expression of fibronectin. Champman et al[6] identified and isolated short cDNA sequence tags from thymocyte and prostate cells in mouse under various hormone conditions. Using the technique they have isolated several differentially expressed sequence tags (DESTs) from the mouse thymocyte cell line, two of these DESTs are rapidly induced by dexamethasome within 2 hours of treatment. From androgen-modulated rat vernal prostate tissue. they also isolated the DESTs.

3.3. Animal Pathogenesis and Cell Physiology.

Chao et al[5] used the Differential Display technique to identify the differential expression between the spontaneous hypertension rat (SHR) and Wistar-kyoto rat

(WKY) in vascellum smooth muscle cells and cloned the special cDNA fragment. To identify unique gene products that could be crucial for circadian and light-dark regulations. Gauer et al[8] have investigated mRNAs which were isolated from rat pineals and retinus. They found that 15 cDNAs show differential expression and four amplified products were selected, based on higher expression during the subjective night and each was subcloned and sequenced. It was found that one of the four cDNA was determined to have significant identity to a known cell adhesion protein. Hamdan et al[11] used the technique to screen numerous genes to gain insight into the molecular mechanisms of intimal hyperplasia. Approximately 5,000 mRNA species were screened, and 11 candidate clons were obtained, DNA sequence revealed homology of 5 clones to known gene sequences. These homologous genes included an interferon-gamma -induced human gene, α -1 protease, inhibitor gene, human retinoblastoma susceptibility gene, and human creatine kinase gene. In the study of identifying of multiple genes in bovine retinal pericytes altered by exposure to elevated levels of glucose, Aiello et al.[11] obtained more then 20 candidate clones which encoding fibronectin, caldesmon, two ribosomal proteins, rieske Fe. S reductase, autoantigens and tyrosine kinases.

3.4. Animal Development.

In analysis of gene expression in the preimplantation mouse embryo, Zimmermam and Schultz [26,27] used the mRNA Differential Display technique to detect the appropriate changes in the temperal pattern of expression of amplicons by DNA sequence analysis those are the genes of cytokeratin endo A and mitochondrial F-1 ATP synthase. In addition, they identified amplicons those likely represent genes whose expression is greatest during the eight-cell stage, the other in the blastocyst.

3.5. Plant Physiology and Resistance.

Zhang et al[25] used the Differential Display technique to compare differential expression between rice strain 77-170 and mutant salt -resistant strain M-20 in saline stress conditions, 13 special cDNA were cloned in 77-170 or M-20. One of those is homologous to Rab16 gene. In order to get more information about carbon metabolic regulation pathways, Tseng et al[23] used this technique to clone and make sequence analysis of sucrose-regulated genes from rice- cultured cells, six candidate clones were identified and sequenced. Clones SI1 and SI2 were repressed by sucrose starvation while clones SR1, SR2, SR3 and SR4 were induced by surcrose starvation. Nucleotide sequence analysis showed that clone SR2 has 94.8% homology to the SalT gene. In Wu R.'s laboratory, mRNA Differential Display was applicated to conduct the genes analysis which are induced in rice endosperm by GA3, more than 25 cDNA fragments were specially obtained, two possible genes were identified: Ubiqutin carried protein gene and Ca^{2+}-ATPase gene.

As mRNA Differential Display technique has obtained great successes in only three years, we can predict that the technique would make greater development and be applied in a wide-ranging field.

4. CHARACTERIZATION

Although mRNA Differential Display is somewhat similar to RAPD technique, and there are also some reports about RAPD used to identify mRNAs, the characterization of mRNA Differential Display is supereminent to that of RAPD in the following way:

4.1. The primers that RAPD utilizes are single arbitrary oligonucleotide, positive results are small. Although one can change the reaction conditions, for example, adjusts annealing temperature, adopts degenerate bases to amplify more bands, the polymorphism of amplified products of mRNA would be partially lost.

4.2. mRNA Differential Display technique selects sets of 3' primers and 5' primers to amplify mRNA. The selection of 3' primers takes advantage of the polyadenylate tail presenting on most eukaryotic mRNAs to anchor the primer at the 3' end of the mRNA and so that to increase the number of templates and resulting amplified bands. 5'primer is 10-arbitrary oligonucletides, it can promise the length polymorphism of amplified products. So we can name 3' primer as the basic primer of the lengthpolymorphism and 5'primer as determinate primer of the length polymorphism.

In the past, the main ways to study gene expression are proteindimensional electrophoresis and subtractive hybridization. It is difficult to obtain enough pure protein for analysing and cloning its genes by means of dimentional electrophoresis although it has high sensitivity. As to subtractive hybridization, the cost is large and thetechnique itself is more complicated and takes longer time, the reproducibility of experiments is not always ideal. The mRNA Differential Display method has several technical advantages compared to these two strategies:

(1) Simplicity: It is based on two popular molecular biological methods —— PCR and DNA sequencing gel electrophoresis.

(2) Sensitivity: 2 μ g of total RNA will be enough to cover all the anchored oligo-dT primers in all combinations with 20 arbitrary 10-mers. Statistically, this would cover all the mRNAs .

(3) Reproducibility: 90%-95% bands of mRNA display are reproducible from run to run.

(4) Versatility: More than two RNA samples can be compared at one time and both dominant genes or recessive genes can be detected simultaneously.

(5) Fast: Within two days the pattern of mRNA display can be obtained. Reamplification of probes and Northern blot confirmation take another week. Most importantly, the assay may be checked at each step[9,15].

However, there are some limitations in the mRNA Differential Display technique. Firstly, in materials which are compared, they should differ only on the object genes and the other genes should be homologous. Secondly, there are other important extranucleic DNA system ----mitochondria and chloroplast which control some activities and characteristics of living process in eukaryote, such as respiration, male sterility, photosynthesis. Primary designed mRNA Differential Display technique is not suitable for identifying extranucleic mRNAs because of lacking the 5' boundary of a poly(A) tail for reverse transcription, although it could be used simplicity, sensitivity and reproducibility to visualize the altered nucleic genes expression in eukaryote.

5. PROSPECT

GenHunter Corporation has developed two kinds of reagent kits for the application of mRNA Differential Display. One kit is composed of the first generation primer, containing 12 two-base anchored oligo-dT primers and 20 10-mers arbitrary primers. Another kit features the third generation of mRNA Differential Display using 3 one-base anchored oligo- dT primers containing HindIII restriction site and 8 arbitrary 13-mers with HindIII restriction site. Amplified cDNA fragments cloned into the PCR-TRAP system could be easily excised by Hind III digestion. Furthermore, it also developed the Message Clean Kit, cloning and sequencing system for obtaining a good quality RNA and accomplishing the successive study related to this technique. These commercialized kits are useful and very convenient for laboratory work.

However, this technique raises a potentially serious problem with regard to the use of isotope as a label for differential display. The decomposition of isotope into a volatile product during PCR and escape of the radioactivity from the tops of the PCR reaction tubes could be hazardous to researchers. If we can establish an non-isotope labelling technique during PCR amplification which also suitable for sequencing gel

electrophoresis with high band resolution, that will improve the safety of this method.

As to the respect of its application, because of several technical advantages as compared to subtractive and differential hybridization, mRNA Differential Display should find wide-ranging and rapid application in the future in molecular basis of hormone regulation, cell differentiation, growth and development, pathological changes, responsing to stress as well as isolating and cloning specific genes.

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发展中的mRNA差别显示技术

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摘要:

随着PCR技术的出现(1985)，在分子生物学界又相继出现了两个很有影响的新技术——RAPD技术(1990)和mRNA差示法(1992)，前者用于分子标记，后者用于基因分离。

mRNA差示法的生物学基础是基因的差别表达，既:单个细胞中表达的基因仅占基因总数的15%。这种基因的差别表达决定了生命的所有过程，如:发育和分化、对逆境的反应、细胞分裂、老化等。图一给出了该方法最初的技术路线。提取要比较的两种或两种以上样品的mRNAs，分别逆转录成cDNAs，经过PCR扩增后，直接进行测序胶电泳即可识别有差别的mRNA。其中、关键的是PCR扩增时两个引物的设计。3'端引物Oligo(dT)MN很容易与具有N'M'-poly(A)-3'末端的大多数mRNA结合，进行cDNA的逆转录合成。M、N提供锚定位点，防止3'端引物在 poly(A) 序列不同位置上的随机结合。5'端为10个碱基的随机引物。这个经验上的碱基数值较理论的6-7个碱基(表一)更能满足测序胶电泳要求的条件:分子大小在500bp左右，每条泳道上条带数在100条左右。