Preparation of probes which lead to the Identification genes that are differentially expressed with retinoic acid (RA) treatment in early Xenopus laevis embryos.

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Abstract:

Total RNA from both treated and untreated stage 14x.laevis embryos was extracted and analysed by DDRT-PCR. The RNA was coverted into cDNA by reverse transcription-PCR by using oligo (dT) primers and amplified by arbitrary 5'primers. The PCR products were compared side by side and analysed on 6% DNA sequencing gel and autoradiography. Plasmid DNA sequencing of cloned cDNAs were determined for one differential fragment (M-band). M-fragment sequence

(279bp) which was isolated from RA-treated stage 14x.laevis cDNA was tailed with poly (A) characteristic of 3'untranslated region and no significant open reading frames were present. A search of the EMBL/Gen bank databases using FASTA program appeared that the 3'end of M-cloned (86bp) has 81% homology to the xenopus laevis cyclin-dependent kinase 2 promoter (CDK2). The reverse transcription-polymerase chain reaction (RT-PCR) analysis appears to support the idea that the quantity of M-mRNAs is increased in the presence of retinoic acid. The 279bp of M-clone was stored at -20˚C to use it as a probe for screen a cDNA library in future.

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Key words: Retinoic acid, Xenopus embryos, M-probe.

Introduction:

Retinoids are a family of low molecular weight, hydrophobic moleclues

derived from vitamin A. Retinol (vitamin A) and its derivatives retinoic acid (RA) are essential in the control of epithelial cell growth and cellular differentiation.

The sequential treatment with activin and retinoic acid can induce the presumptive ectoderm from Xenopus blastula to differentiate into a morphological and functional pancreas in vitro (Naomi et al., 2000).

Blumberg et al. (1996) reported that the bioactive retinoid in the Xenopus egg and early embryo is capable of binding to and transactivating retinoic acid receptors (RARs). The differential activation of the three RAR-mRNA (α, B, Y) were studied in mouse embryos and placentas using reverse transcriptase-polymerase chain reaction technique (Tsung-Chieh J. Wu et al., 2005). The results showed that RAR genes were the important regulators during early embryogenesis. Recent studies by

Muriel Rhinn and Pascal Dollel (2012) indicate that RA acts as ligand for nuclear RA receptors, converting them from transcriptional repressors to activators.

Retinoids and their receptors regulate gene expression and morphogenesis of the eye

(Panagiotus A. Tsonis et al.,2002), its intraretinal RA metabolism distribution and

expression in vertebrate eye regulated by Vax2 eye-specific homeobox gene (Giovanna Alfano et al., 2011).

RA has been detected endogenously in different vertebrate embryonic fields.

Lisa L. Sandell et al., (2007) determined that the retinol dehydrogenase RDH10, is critical for the spatiotemporal synthesis of RA.

It is present during gastrulation in zebra fish (Costaridis et al., 1996) and Xenopus (Chen et al., 1992; Creech kraft et al.1994) embryos and in Hensen's node of the chick embryos (Chen et al., 1992), and RA is synthesized from retinol in the node of the mouse embryo (Hogan et al., 1992). In pregnant mice, a single oral dose of retinoic acid on day 11.5 of gestation failed to induce histological changes in fetal epidermal development (Rosa A. Garcia-Fernandez et al., 2006).

Several lines of experimentation suggest that endogenous retinoids, play a role in the anterior / posterior development of the central body axis and the limbs of vertebrates.

Teratogenesis studies suggest that both retinoid excess and deficiency are capable of disrupting the development of these structures (Means and Gudas, 1995). Kirsten

M. Spoorendonk et al. (2008) observed that RA causes increased activity of axial

Osteoblasts, resulting in defective skeletogenesis.

Ascidian embryos treated with RA exhibited truncated phenotypes in a dose-dependent manner similar to the anterior truncations seen in vertebrate embryos (You Katsuyama et al., 2003). Sussie Dalvin et al. (2004) observed an RA-dependent reduction in fetal lung mesenchymal proliferation.

The differential screening technique is one from the effective methods needed to identify and isolate genes that are differentially expressed in various cells or under altered conditions. This technique is used to identify genes which show quantitative, variation between two cell types. It allows genes which show even a small differential expression to be compared and isolated. Current methods to distinguish mRNAs in comparative studies rely largely on the subtractive hybridization method

(Lee et al., 1991). The subtractive hybridization approach of RA-treated embryonal carcinoma cells (EC) led La Rosa and Gudas (1988a) to identify an early retinoic acid-1 (Era-1) gene. By using this technique, Philippe Bouillet et al., (1995) also characterized a novel RA-inducible, Stra1 (LERK-2 / Eplg2). An alternative method to differential hybridization technique has been developed by Liang and Pardee (1992). The RT-PCR analysis revealed by Ina State et al. (2009) that the retinoic acid is an important morphogen that regulates the development of central nervous system. This method was termed differential display reverse transcription-PCR (DDRT-PCR). It can be used for three purposes. One is to visualize mRNA compositions as short cDNA bands. Second, these cDNAs can be quickly sequenced, and compared with sequences in data banks. Third, individual bands can readily be cloned and used as probes to isolate genes from cDNA or genomic libraries. The essence of this

method is to use a set of oligonucleotide primers, one is an anchored oligo-dT primer and the other is short and has an arbitrary base sequence. This differential display strategy was the objective that applied in this paper to total RNA extracted from untreated and RA-treated x.laevis embryos, this to identify fragments of cDNA that are differentially expressed with RA treatment and could be used as probes in future work.

Material and methods:

Eggs and embryos:

Eggs were obtained by injecting Xenopus laevis females with 250 units of human chorionic gonadotrophin B 16 hours before laying. In vitro fertilization and embryo culture were done in 1/10 x BarthX (88mM NaCl, 1mM KCl, 2.5mM $NaHCO_3$, 15mM Tris-HCl pH7.6, 0.3mM $CaNO_3$, 0.41mM $CaCl_2$, 0.82mM

$MgSO_4$). Staging was according to Nieuwkoop and Faber (1967).

Isolation of RNA from X. laevis embryos:

Total RNA was extracted from untreated and RA-treated stage 14 x.laevis embryos. The treatment with retinoic acid was as mentioned in results and discussion. Each 20 embryos were homogenized in 1ml mixture of NAE (0.3M Na Acetate pH6.5, 1mM EDTA) containing 2% SDS and neutral phenol at 4˚C and incubated 5 minutes at room temperature. Following its isolation, RNA was treated with DNAse I (Boehringer Mannheim) to remove contaminating DNA. The RNA was extracted with phenol/chloroform, ethanol precipitated by $1/10^{th}$ volume of 3M sodium acetate pH 5.6. The RNA pellet was washed in 70% ethanol, dried in vacuo and dissolved in DEPC treated water at final

concentration 1µg /µl. RNA concentration was estimated spectrophotometrically then split in to aliquots and stored at -70˚C. The total RNA was separated on 1% agarose gel and visualized with ethidium bromide by using a uv transilluminator.

PCR amplification:

The first strand cDNA was synthesized in a 20µl reaction mixture containing 2µg total RNA isolated from treated and untreated stage 14x. laevis embryos, 3.75µM degenrate primers, MMLV-reverse transcriptase, reverse transcriptase buffer (50mM Tris pH 8.0, 30mM KCl, 5mM $MgCl_2$), 1mM DTT, 10 units RNasin and 50µM dNTP mix. The reaction was incubated at 37˚C for 60 minutes. Two µl first strand cDNA was used as a template in the next PCR reaction. The second strand cDNA reaction was included: 2µl of 10 x PCR buffer (50mM KCl, 1.5mM MgCl, 10mM Tris-HCl, pH8.3), 2µM dNTP mix, 0.5µM 5' arbitrary primer, 2.5µM degenerate primers mix, 0.5µCi α-^{35}S dATP and 1 unit of Taq DNA polymerase. During the 40 cycles of the reaction, the hot start was 94˚C/1.5 minutes and the Taq DNA polymerase was added at 72˚C. The denaturing, annealing and extension steps were optimized for 30s at 94˚C, 42˚C and 72˚C, respectively. A final elongation step at 72˚C for 5 minutes was added. The PCR products were compared side by side on a 6% DNA sequencing polyacrylamide gel and autoradiography. The differential M-band was excised from dried polyacrylamide gel and purified. That the cDNA of this bands was defused by boiling the gel slices and soacked in an extraction buffer

(0.5M ammonium acetate pH4.6, 0.1mM EDTA, 1mM Mg Cl_2 and 20µg tRNA) overnight. The cDNA was washed in 70% ethanol, resuspended and introduced into a reamplification PCR reaction without α-^{35}S dATP.

The PCR product of interest (M-cDNA) was agarose gel isolated and subcloned in a pGEM-T vector.

RT-PCR techniques:

In RT-PCR, total RNA from untreated and RA-treated stage 14x. laevis embryos was extracted as described by CLONsep Protocols. 0.5µg of total RNA was heated to 75°C for 5 minutes and used as a template for first strand cDNA synthesis in the presence of 3.3µM random hexanucleotides as primers. The total reaction (30µl) was incubated for 60 minutes at 42°C, then heated to 95°C for 5 minutes and stored at -20°C. Two µl of the above cDNA synthesis was used for each 50µl PCR. The PCR was carried out with 1µM left and right specific primers, dNTPs (200 µM) and 1µCi α-^{32}P dGTP. Cycling parameters were: one cycle, 94°C/3 minutes, 55°C/1minute, 72°C/1minute followed by: 20 cycles, 94°C/30secs., 55°C/1minute, 72°C/1minute, and final polymerization step was 72°C/5minutes.

Transformation of competent cell:

E.coli cells were grown a 20 ml culture of L-broth at 37˚C with agitation to an OD600 of 0.5. The exponentially growing cells were centrifuged and the pellet was resuspended in 1/2 original volume of ice cold 100mM Mg Cl_2. After centrifugation the pelleted cells were resuspended in 1ml of fresh ice cold 100mM CaCl2, then divided into 100µl alquots of competent bacteria.

0.1ml of competent cells were mixed with the differential cloned cDNA and placed on ice for 30 minutes with intermittent mixing. The cells were heat-shocked at 42˚C for 2 minutes then diluted to 1ml with L-

broth. Cells were incubated for 60 minutes at 37˚C and then pelleted and resuspended in 100µl of L-broth prior to plating on appropriate L-Agar plates for overnight incubation at 37˚C.

When the transformed cells were prepared, the method of large scale plasmid DNA isolation (Birnboin and Doly (1979) was applied. The recombinant plasmid cDNA was further purified by Cs Cl density gradient centrifugation (Maniatis et al., 1982) and Ethidium bromide was removed by repeated extractions with isoamyl alcohol. The aqueous phase was dialysed against several changes of TE (10mM Tris-HCl (pH7.5), 1mM EDTA (pH8)). The sequence of the subcloned cDNA was determined using the commercially available sequenase Kit and following the manufacturer's protocol.

Results and discussion:

Existing methods to separate mRNA in comparative studies rely mostly on subtractive hybridization. This technique has been applied in isolating a number of RA response gene (Jonk et al., 1994; Bouillet et al., 1995). Liang and Pardee

(1992) originated a modern process that allowed detection of expressed genes in different cell types. This method, named differential display reverse transcription polymerase chain reaction (DDRT-PCR), was used to separate and clone individual messenger RNAs by means reverse transcripts.

Embryo treatment:

In order to characterize genes that effected by retinoic acid in Xenopus laevis, separated Xenopus embryos were transferred to barthX with or without 10M RA

(10ul 10^{-2}M RA in 10ml BarthX). The total RNA from both treated and untreated embryos was extracted and analysed by DDRT-PCR:

1) controls- no treatment.

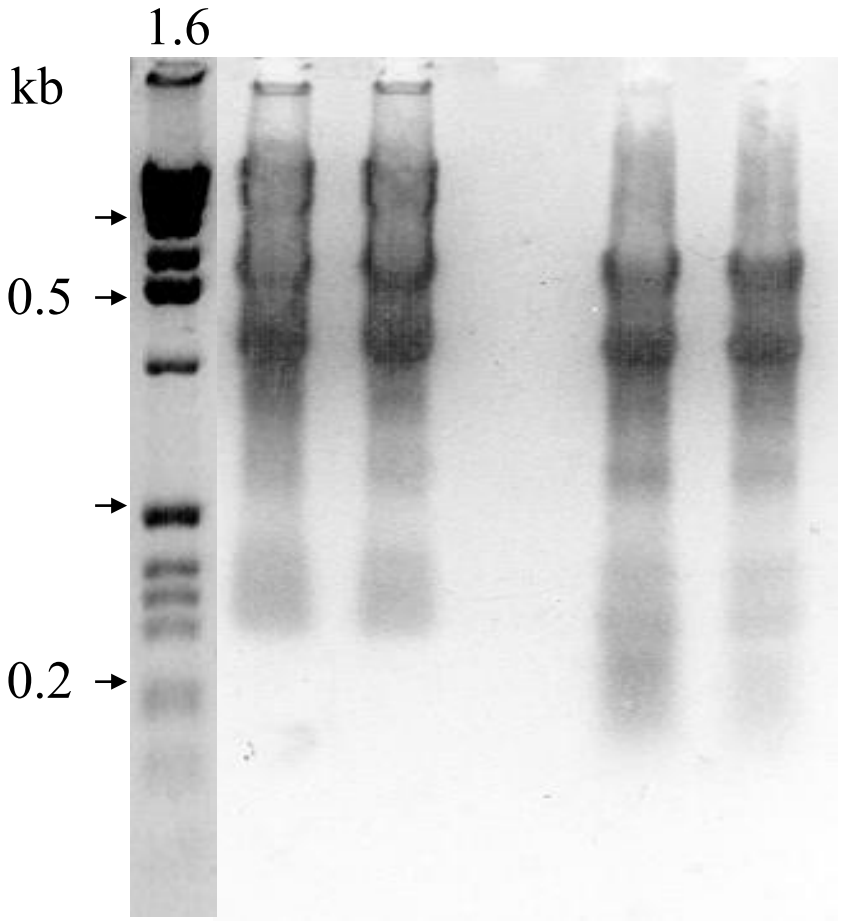
2) retinoic acid treated embryos.

The duration of exposure to RA was 3 hours after stage 9 and the experiment was carried out at 14˚C.

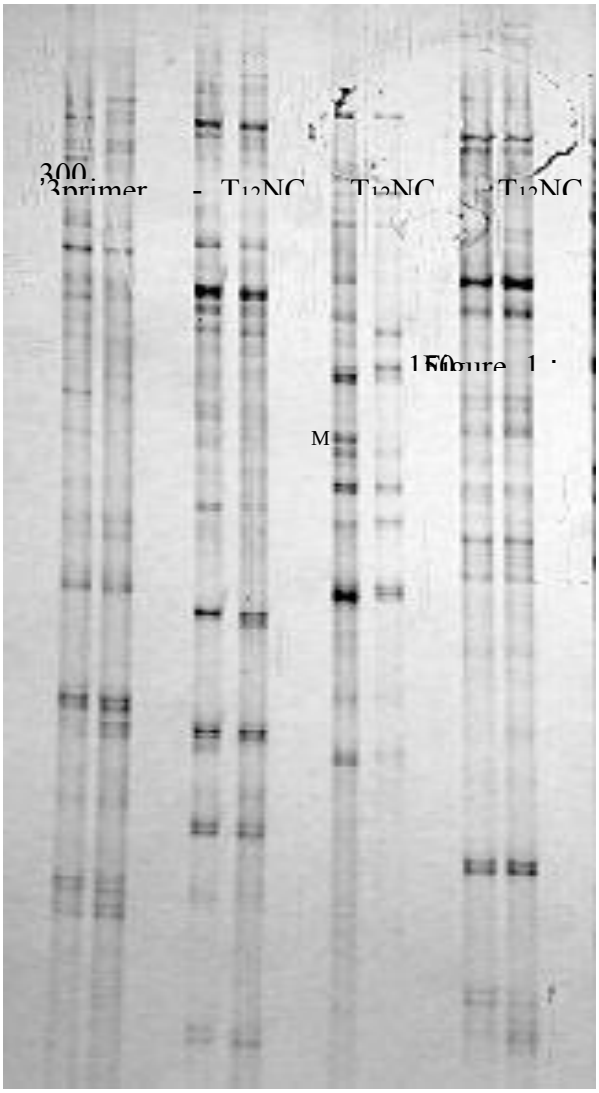
Differential display RT-PCR:

The total RNA, isolated from treated and untreated stage 14 Xenopus embryos (figure 1) was converted into cDNA by reverse transcription-PCR. The reaction was done in different independent reactions using oligo (dT) primers : T_{12}NG, T_{12}NA,T_{12}NC,T_{12}NT (where N may be dG, dA, or dC). After 60 minutes at 37˚C the sample was added to 20 ul of PCR labeling mix (see materials and methods) and amplified by arbitrary 5'primers (501: 5’-ATACAGCAGG-3’, 502: 5’-ACAGACTGAC-3’, 503: 5’-ACGATACACG-3’ and 504: 5’-AGACTTCGAG-3’) in combination with the same above oligo (dT) primers.

The only 3’primer that has been worked with the above 5’primers was the degenerate primer T_{12}NC. The reaction was tried again by using T_{12}NC primer in all differential PCR reactions performed. The PCR products were compared across the


1.6
kb
0.5
0.2

3.0



bp

treatment samples,side-by side, and analysed on a 6% DNA sequencing gel and autoradiography (Figure 2). The remaining three 3'primers ($T_{12}NG$, $T_{12}NA$ & $T_{12}NT$) generated a smear down the gel with no real distinct bands. The failure of these primers to give distinct banding patterns may be due to the poor specificity of annealing to the

untranslated regions of mRNAs in the initial reverse transcription. The use of poly (A^+) mRNA instead of tatal RNA in the reverse transcription reaction could optimise the reaction conditions for these primers in order to obtain better differential display results.

The primers that produced fine banding styles varied in the number of marked bands present in a lane. In my attempts the number of bands varied from 17-75 from a track, the size of these fragments was in the range between 100-400 bases. The greater number of these bands were appeared at a equivalent intensity across the two treatments.

Cloning and sequencing differential products:

The differential bands (M, K, H, Y) from treated and untreated mRNA were recovered and reamplified in 40ul reaction volume using the same primer set and PCR conditions as used in the mRNA display except the dNTP concentrations were at 20µM instead of 2-4µM and no isotope was added (figure 3). The size of fragments is inbetween 150 to 350bp.

The PCR fragments obtained were then gel separated and cloned into two different vectors. Y-fragment was cloned in HincII site of pBluescript (KS), this after blunting the ends of the PCR products by using Klenow. Other three fragments

(K, H & M) were ligated into the T-vector using the TA cloning system (figure 4). Plasmid DNA sequencing of cloned cDNAs were determined for two differential fragments (Y& M). The sequence obtained in each case was tailed with poly (A), characteristic of 3'untranslated region and no significant open reading frames were present (figure 5).

A search of the EMBL / Gen bank databases using the FASTA program

(Pearson and Lipman 1988) failed to bring up any significant

nucleotide matches for the Y-clone. The homology of the M-clone (figure 5A) was to the Xenopus laevis cyclin-dependent kinase 2 promoter (CDK2). The 3'end of M-cloned (86bp) appeared to has 81% homology to cDK2). The cell cycle is activated by the synthesis and destruction of their cyclin regulatory subunits

(reviewed in King 1994). The remaining part of the sequence (195 nucleotides) is related to various previously identified proteins like: lamins (A, B, C) and Homo sapiens retinoic acid receptor alpha (RARα).

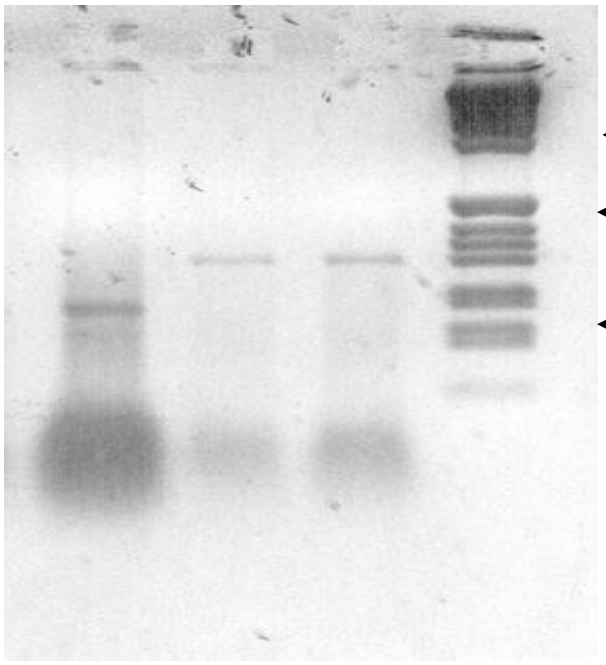
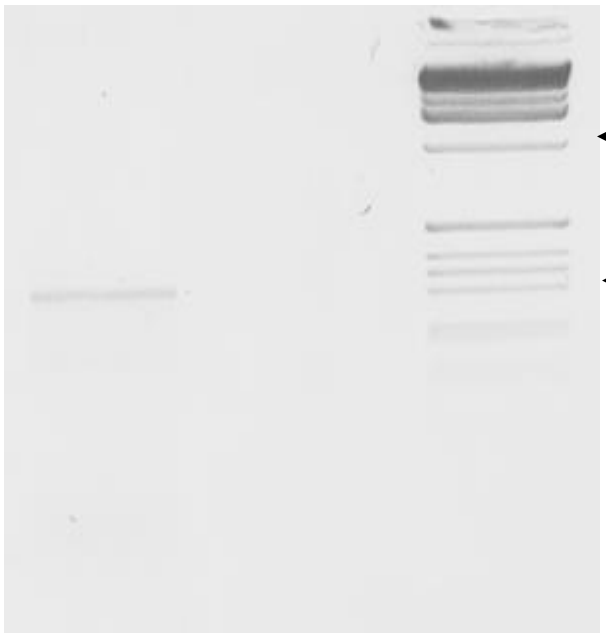
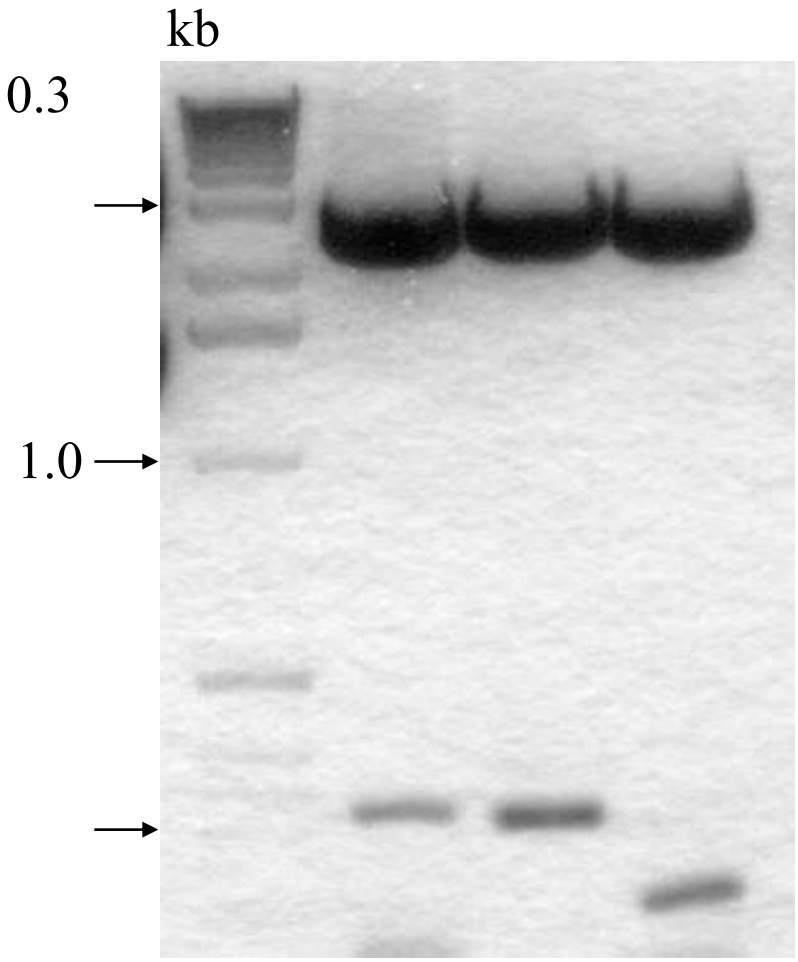


Figure: 2 .
PCR display-:
Y Comparative analysis of
stage 14 *Xenopus laevis*
K indicated above the lanes
Lanes 2, 4, 6, 8: RNA ex
H and Y) that are selected
H



kb



A

TTTTTTTTTTTTTCCGGGCAGTAAATGGTTATGAACCCCCAGG

*

TTTTAGGATCCCGGCCTCCGTCCCCCCCCAGGAATGCTTCCGCTTA

GTGTTATGAGACGGAGACTTTCATTAATTATTTATATTTTTTTTC

GGCATAAGAACAAAACTTGCTACGGTTTAAACCACTTTGCTGCCT

CTCCTCTCCCCGGCAGACATGGGGTTAAAAATGATGGTTGATCCC

*

AATGTTATTAATAGGGGAAAAAAAAAAATAAAAACCTGTTGGGA

AAAAAAAAAA

B

TTTTTTTTTTTCCCATTTGCAAAGTGAGGCACAACCAAGTCAA

AATCATATCCCTGGGAGAGTCAGTTTCTGGTTTCCTTCTCACG

AAAGCTGCGGTACATATTACAAGGTCATCTAAGGGAATTGCAT

AATGAAGATTACATGAAAGGGATGCACAGCCTGCTTACGGAAT

CGGTATTAATGCCAGCTAGTCGCCCCTAAATTAACTACGGCAT

CACCAATTGGAGGTGAAACACCAAATGGCTCATCAACTCC

Figure: 5.

Nucleotide sequences of M- and Y-clone.

(A) M-fragment was isolated from retinoic acid treated stage 14 X. laevis cDNA. The sequence which between the two stars (below the nucleotides) represents the nucleotides that are identical to x14K-gene sequence (full length paper).

(B) Y-fragment was recovered from the differential display acrylamide gel (figure 2), purified and cloned in a pBluescript (KS). The mentioned fragment was from untreated stage 14 Xenopus laevis cDNA .

The sequences of M- and Y-clones suggests that both of them were from a 3' untranslated region. The sequences selected for primer design are shown underlined.

RT-PCR to comfirm differential expression of cloned products:

Reverse transcription-polymerase chain reaction (RT-PCR) assays were carried out to study the RA-inducibilty of the M- and Y-cloned in stage 14x.laevis embryos.

RT-PCR was carried out as mentioned in methods. First-strand cDNA was synthesised from 0.5ug of each treated and untreated total RNA, with random hexanucleotides as primers.

The 5'extension of the cDNA was obtained with each of Y-fragment pecific primers:V5'-AAGTGAGGCACAACCAAGTC-3' and S5'-TGGTGATGCCGTAGTTA-3' and M-fragment specific:- primers: X5' CCGCTTAGTGTTATGAGACG-3' and M5'-GGATCAACCATCAT-3'.The primers were designed to produce 140bp and 199bp from M and Y respectively

(figure 5). Figure 6 shows that after 20 cycles of PCR, M-transcript was easily detected in the RNA from treated embryos but it was not detectable in the untreated. The treated track shows a high level of expression, comparable to the untreated one. This level of expression is clearly due to the presence of retinoic acid and therefore supplies strong evidence that

the expression of this gene is upregulated by retinoic acid. Surprisingly, however, the M-produced band was larger than the expected 140bp, estimated at about 847bp. The explanation for difference from the expected size is probably due to a rearrangement having occurred in the creation of the M-sequence. That this size which is in between the M-primers in RT-PCR assay was consistent to the distance between the same primers in the x14K gene (full length paper).

The intensity of the detected Y-band, both in RA-treated and untreated RT-PCR reactions indicated that it could be repressed with retinoic acid.

The linearity of the RT-PCR assay was checked out by subjecting different quantities of retinoic acid-treated stage 14 X. laevis cDNAs to RT-PCR analysis. The specific primers of M-clone were used and the result was as demonstrated in figure 7.

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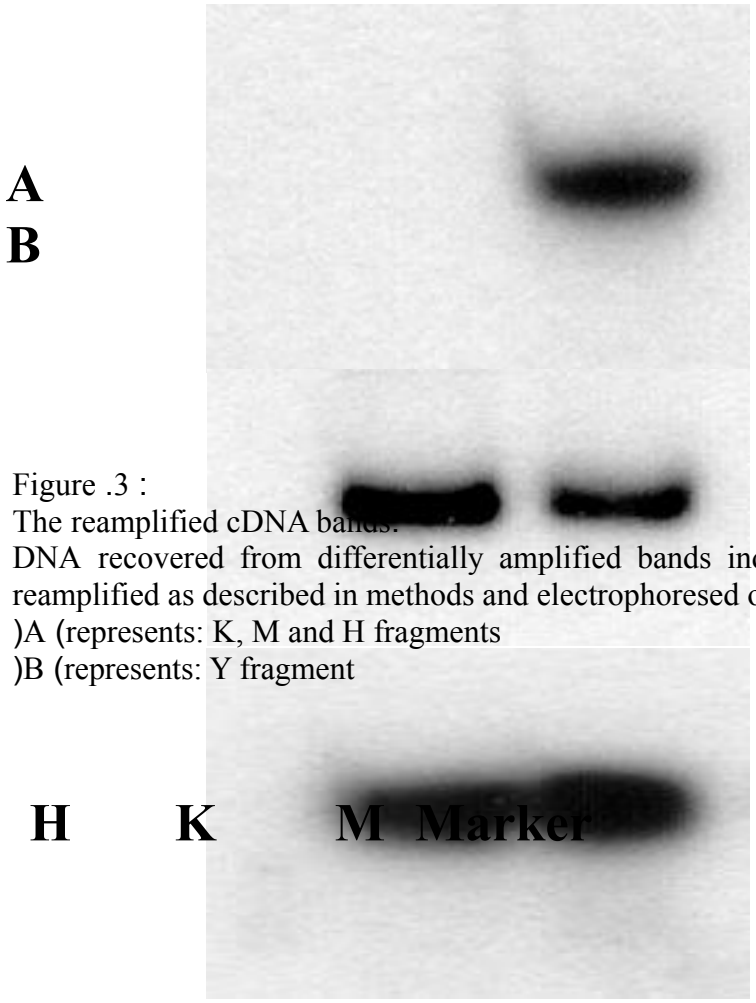
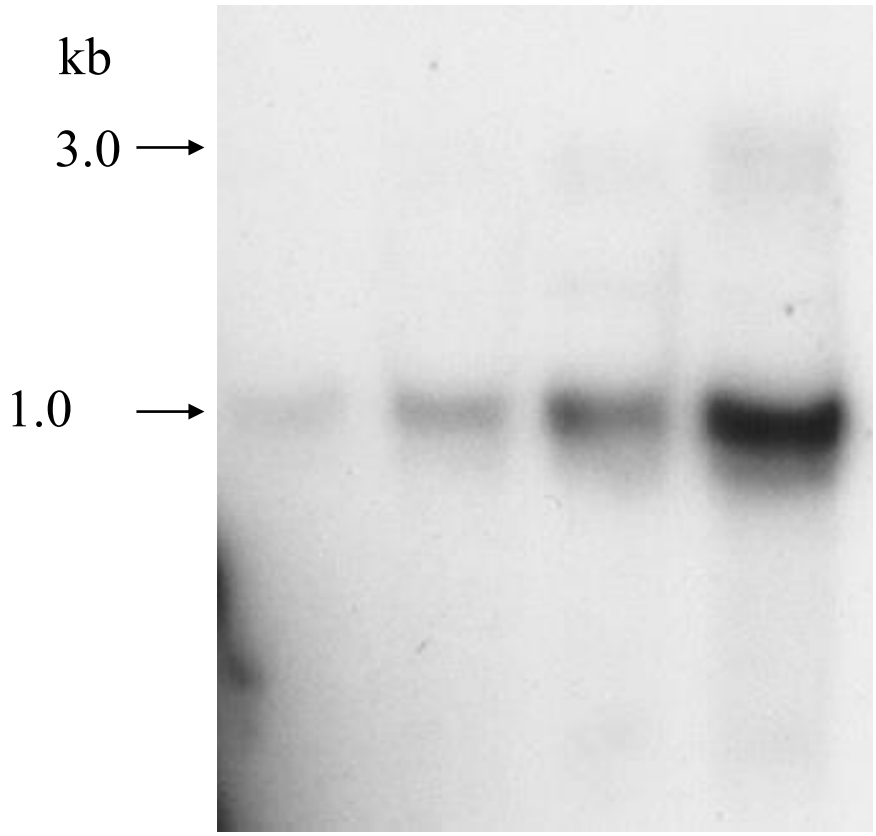


Figure .3 :
The reamplified cDNA bands.
DNA recovered from differentially amplified bands indicated in figure 2 was reamplified as described in methods and electrophoresed on a 1.5 % agarose gel.
)A (represents: K, M and H fragments
)B (represents: Y fragment


kb
3.0
1.0
0.3

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المستخلص العربى:

أعداد مجسات (probes) لجينات متأثرة بحمض ال Retinoid المعالج في أجنة الضفدعة الأفريقية (Xenopus laevis)

Retinoids هو من عائلة ذات الأوزان ألجزيئيه الخفيفة والمحبة للماء (hydrophobic) و ألمشتقه من فيتامين A. حمض ال retinoid هو من العوامل المهمة والمتحكمة في التمايز الخلوى و نمو الخلايا الطلائية .

العديد من العلماء لاحظوا إن النشاط الحيوي لل retinoid في بيضة الضفدعة وأجنتها المبكرة قادراً علي الارتباط بمستقبلاته (retinoic acid receptors) المحفزة.

حمض ال retinoid اكتشف داخلياً(endogenous) في مراحل مختلفة من أجنة الفقاريات . العديد من التجارب اوضحت ان ال retiooids له دور في النمو المحوري المركزي وأطراف الفقاريات . الدراسات اوضحت ان زيادة ونقصان ال retinoid قادرة علي أحداث خلل في نمو تلك التراكيب العضوية.

من هنا جاء التفكير في الحصول علي مراحل مختلفة من أجنة ال Xenopus laevis و معالجتها بحمض الretinoids ومقارنتها بمجموعة أخري غير معالجة . وباستخدام انواع التقنية منها طريقة العرض التمايزي (differential display) و المتضمنة لعملية ال-PCR و المشتمله علي oligo(dT)primers و primers أخري منتقاه. طبقت الطريقه علي RNA الكلي والمستخلص من كل من الاجنة المعالجة وغير المعالجة وأدت الي التعرف علي أنواع ال- mRNAs المتمايزه في النسخ عند المعالجة بال retinoids .

فبأدوات الهندسة الوراثيه قمت بعزل حزم (bands) متمايزة من cDNAs حيت تم حفظها (cloned) داخل نواقل (Vectors) من البلازمد. الحزم المتمايزه تم عرضها و تصويرها باستخدام كل من polyacrylamide-gel & -agarose. حيت بعد ذلك تم تنقيتها والاكثار منها باستخام البكتيريا E.coli.

باستعمال ال primers المناسبة وبعملية ال- sequencing تم التعرف علي انواع القواعد المكونة لسلسلة قطع cDNAs المتمايزة والمتأثرة بال- retinoids.

تم تحليل قطع ال cDNAs من حيث وضعيتها في جيناتها بين النهايتين 5' end & 3' end.
كذلك تمت مقارنة التسلسل القاعدي للقطع بالبحث فى ال- Gene bank data bases والحصول علي نسبة كبيرة من التجانس القاعدى مع جينات أخري في الضفدعة الأفريقية.

نتائج تجارب النسخ العكسي لل- PCR في كل من ال- RNA الكلي المعالج وغير المعالج عند استخدام ال primers السداسية العشوائية (random hexanucleotides)لاول خيط cDNA مع مشاركة primers خاصة (specific primers) صممت من التسلسل القاعدي للقطع المتمايزة لتكوين الخيط الثاني لل-cDNA, دعمت فكرة ان كمية mRNAs للقطع المتمايزة تزداد وتنقص عند وجود ال retinoids.

فبهذه النتيجة يمكن استخدام هذه القطع المتمايزة في كل من cDNAs المعالج وغير المعالج كمجسات (probes)لايجاد التسلسل القاعدي الكلي بالنظائر المشعة (full length coding region) لها كجينات منفصلة عندما يتم المسح بها(screening)في مكتبة ال- cDNA الخاصه والمنتمية إلي الضفدعة الأفريقية (Xenopus laevis cDNA library).