The full length of gene (x14K) that differentially expressed with retinoic acid (RA) treated Xenopus laevis embryos

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Abstract

The differential display RT-PCR technique has allowed me to isolate a gene whose expression is upregulated by retinoic acid in stage 14 x.laevis embryos. The M-band pattern which are generated from this strategy (probe paper), was appeared to be induced by retinoic acid. This M-cDNA fragment was used as a probe. The full length cDNA clone encoding region of this probe-fragment, termed x14K-gene was isolated from a kidney xenopus cDNA library.

Sequencing of x14K-gene generated a continuous sequence of 2271 bp that contained an open reading frame of 1284 bp. Expression of x14K-gene in rabbit reticulocyte lysate produced a protein of the predicted size (47 kD) as determined from the largest open reading frame. A search of the GenBank database has identified that x14K-gene is more closely related to a *Xenopus* mitotic phosphoprotein MP43 than any other previously identified proteins. The two *Xenopus* proteins share 84% identity. Most lamins (lamin B1, A, C & C2) which were observed to be substrates of a family of cyclin-dependent kinases (CDKs), apppeared to have homology to the carboxy end of x14K-gene.

Key words: Retinoic acid, Xenopus embryos, x14K-gene.

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Introduction

Retinoic acid is a derivative of vitamin A. The main sources of vitamin A in the diet are provitamin A carotenoids from vegetables and retinyl esters from animal tissues. Several lines of experimentation suggest that endogenous retinoids, play a role in the anterior/posterior development of the limbs of vertebrates. The first observations relating to the control of the A-P axial pattern of the limb were those of Saunders and Gasseling (1968). Studies by Tickle et al. (1982), Summerbell (1983) and Maden (1985), showed there was a duplication of digits in the chick limb receiving retinoic acid. Retinoic acid has profound effects on the determination of many embryonic tissues, including mesoderm and ectoderm (Durston et al., 1989; Sive et al., 1990; Ruiz I Altaba and Jessel, 1991a; Amanda Janesick et al., 2012). It is essential for patterning the endoderm of pharyngeal arches (O. Wending et al., 2000) and is essential for pattern formation and differentiation during Zebrafish embryogenesis (Johanna M. Lampert et al., 2003). Durston et al. (1989) found that treating early xenopus embryos with RA causes microcephalic embryos which lack most or all of the forebrain and midbrain as well as the sense organs and is induced axial truncation during embryonic stages (Isabel olivera-Martinez and Kate G. Storey, 2007). RA has a novel role in patterning the avian forebrain during presomite stages (Aida Halilagic et al, 2003). In the same time, retinoid production is required in the embryonic mouse telencephalon (Ronald R. Waclaw et al., 2004).

How these simple molecules can exert such effects during embryogenesis and in the adult is an area of intense investigation.

The differential hybridization technique (Lee et al., 1991) was a very useful method of analysis. It has been applied to obtain novel genes

whose mRNA expression levels are altered by exposure to retinoic acid in embryonal carcinoma cells (EC). RA-treatment of EC cells causes the modulation of expression of a large number of genes implicated in early development, including members of the RAR, Hox, Wnt, CRABP, and CRBP gene families (LaRosa and Gudas 1988b; Schuuring et al., 1989; Wei et al., 1989; Jonk et al., 1992). An alternative method to differential hybridization techniques has recently been developed by Liang and Pardee (1992). This method was termed differential display reverse transcription-PCR (DDRT-PCR). The latter method was used by Mason et al. (1996) to isolate a RA-induced gene (x17C) from stage 13 x. laevis embryos and used by Brennan H. C. et al. (1999) to determine the inducibility of the pronephric glomus of Xenopus laevus in the presence and absence of RA. In this paper the treated retinoic acid M-cDNA fragment that was extracted from the differential display gel (probe paper) was randomly labelled with α-(32p)dGTP and used as probe to screen a kidney x. laevis cDNA library.

Material and Methods

Preparation of recombinant M-cDNA

The differential retinoic acid M-cDNA fragment (Probe paper) was cloned into the PCR-1000 plasmid vector (Invitrogen) as outlined in the TA Cloning Instruction Manual K2000-01. The insert was amplified by using the method of large scale plasmid DNA isolation as described by Birnboin and Doly, 1979. Plasmid DNA was further purified by CsCl density gradient centrifugation (Maniatis et al., 1982). Plasmid bands in the 37ml Beckman quickseal tubes were visualised under long-wave ultraviolet light and removed with an 18 gauge needle. Ethidium bromide

was removed by repeated extractions with isoamyl alcohol. The aqueous phase was dialysed against several changes of TE.

The purity and concentration of the recombinant M-cDNA sample was spectrophotometrically determined and the intergnity of the cDNA was onfirmed by agarose gel electrophoresis and visualized after ethidum bromide (EtBr) staining.

Labelling of M-cDNA probe

The amplified 279bp McDNA insert was randomly labelled with α-[32-P]dGTP as mentioned in Amersham random priming Kit (Feinberg and Vogelstein, 1984). 50ng M-cDNA was heated to 95˚C for 3 minutes and subsequently cooled on ice. The denatured M-cDNA was mixed with 10µl OLB, 2µl BSA (10mg / ml), 5µl (50µCi) labelled dGTP and 2µl of klenow in final volume of 50µl. The mixture was incubated overnight at room temperature and the reaction was stopped by addition 2µl 0.5M EDTA pH8.0.

Plating the cDNA library

A kidney x.laevis cDNA library was constructed by Stratagene Company. The library was generated using the Gigapack II Gold packaging extract and synthesised using the ZAP-cDNA synthesis method. The linker-primer was designed with a GAGA sequence to protect the XhoI restriction enzyme recognition site and 18-base poly (dT) sequence. The restriction site allows the finished cDNA to be inserted into the vector unidirectionally in the sense orientation with respect to the lacZ promoter. The packaged cDNA library was plated out onto 150mm x 15mm NZY plates using the host strain XLI-Blue at approximately 200.000 plaques per plate and were incubated at 37˚C for 8 hours. The plaque lift

procedure was used to transfer bacteriophage DNA from plates to Nylon filters (Hybond N) and is essentially as described by Benton and Dans (1975). The filters were backed at 80˚C for 2 hours in a vacuum oven prior to hybridization with radiolabelled probe.

Probe hybridization

The Nylon membranes (Hybond N, Amersham) were prehybridized for 3 hours in 50% deionized formamide, 6 x SSC, 0.1% (w/v) SDS, 5 x Denhardt's solution and 100µg /ml denatured salmon sperm DNA at 42°C. After prehybridization, the hybridization was carried out for 14 hours under the same conditions with 0.05 µg / ml of M-cDNA probe. Filters were washed for 30 minutes 2 x SSC, 0.1% (w/v) SDS at room temperature, followed by 20 minutes in 0.1 x SSC, 0.1% (w /v) SDS at 50°C. The filters were air-dried and autoradiographed at-70°C for overnight to 2 days .

The hybridization-positive plaque was picked from the master plate, replated and screened. This process was repeated until the derived hybridization-positive plaque was pure.. A pure plaque was used to prepare a phage lysate. The phage stocks of the positive clone was stored at 4˚C.

In vivo excision protocol

Recombinant pBluescript plasmid (SK-) contained the full length x14K-cDNA was recovered from positive recombinant Lamda ZAPII clones by *in vivo* excision. This was carried out exactly as described by the manufacturers. 100µl of the excised phagemid supernatant was incubated with 200µl OD600 = 1 SOLR cells at 37˚C for 15 minutes and then plated onto LB plates containing 50µg / 1ml ampicillin and incubated overnight

at 37˚C. A single colony was isolated and the recombinant pBluescript plasmid DNA was prepared and stored for analysing. Plasmids with appropriate size of x14K inserts were sequensed in both directions using the dideoxynucleotide method as outlined in the sequenase version 2.0 DNA Sequencing Kit(USB). The sequences were read and entered into GenBank to determine nucleotide and amino acid homologies.

Preparation of x14K for transcription

Capped synthetic mRNAs were synthesized as described (Krieg and Melton, 1984; Wright et al., 1989). After mapping the x14K restriction map and by using the polylinker of pBluescript (KS) in different stages of cloning, the x14K was cloned in between the E.coRI and NotI sites of pBluescript RN3, to obtain the full length of pBRN3 / x14K in the correct orientation. The pBRN3 / x14K was linearized with SfiI and transcribed with T3 RNA polymerase to yield the x14K mRNA. The reaction of transcription was stopped by adding 15µl of ammonium acetate stop solution (5M ammonium acetate, 100mM EDTA) and extracted with phenol / chloroform. The synthesized x14K mRNA was precipitated with 1 volume of isopropyl alcohol and chilled overnight at -20˚C. After centrifugation, the pellet was resuspended in Rnase-free H2O at 0.56µg / µl and stored at -70˚C.

Cell-Free Translation

3.5µl of x14K mRNA isolated from a kidney x. laevis cDNA library was incubated in nuclease-treated rabbit reticulocytes (Promega) containing 30µCi [35S] methionine. The reaction of translation was Rnased and stopped by addition of 10 x SDS-PAGE loading buffer and analysed by SDS-PAGE and autoradiography(figure 5).

Results

Screening library

The amplified 279bp M-cDNA fragment (figure 1) was randomly labelled with α-[^{32}-P]dGTP (Amersham) and used as probe to screen the cDNA library made from X.laevis kidney in ZAPII. This library was plated as described in methods. Recombinants were transferred to nylon membranes (Hybond N, Amersham), denatured and baked for 2 hours at 80°C. Hybridization was carried out for 16 hours at 42°C. The final wash of membranes were in a stringent wash conditions of 0.1 x SSC at 60°C for 20 minutes and autoradiographed overnight. In the first screening a total of 5 positive-hybridising plaques were obtained. Only one of them was selected of which it gave a strong signal at the third screening (figure 2). This plaque was picked, a liquid lysate was prepared for it. The plaque was converted to double stranded recombinant Bluescript phagemid (SK$^-$) as described by stratagene company.

Analysis of x14K-gene by restriction mapping

Large scale plasmid DNA was prepared for the positive clone and analysed initially by restriction mapping. Standard single and double restriction enzyme digests were used to determine the preliminary restriction enzyme map of full-x14K. It was determined that there was no sites for XbaI, XhoI, SfiI and NotI. The full-x14K had a single site for KpnI, HindIII, SmaI, EcoRI and XmaI, the position of these sites was determined by double digests with a non-cutter located at one end of the clone, in the polylinker. BamHI, and HincII digestion released 3 fragments, indicating two sites for each of them. The sample of the resulting restriction enzyme digests was electrophoresed through a 1%

agarose gel and the size of the fragments was determined as shown in figure 3

TTTTTTTTTTTTTCCGGGCAGTAAATGGTTATGAACCCCCAGGAG

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TTTTAGGATCCCGGCCTCCGTCCCCCCCCAGGAATGCTTCCGCTTA

GTGTTATGAGACGGAGACTTTCATTAATTATTTATATTTTTTTTC

GGCATAAGAACAAAACTTGCTACGGTTTAAACCACTTTGCTGCCT

CTCCTCTCCCCGGCAGACATGGGGTTAAAAATGATGGTTGATCCC

*

AATGTTATTAATAGGGGAAAAAAAAAAATAAAAACCTGTTGGGA

AAAAAAAAAA

Figure:1.

Nucleotide sequence of M-clone which was isolated from retinoic acid treated stage 14 *x.laevis*. The sequence which between the two stars (below the nucleotides) represents the nucleotides that are identical to x14k-gene sequence. The sequences selected for primer design are shown underlined.

FIRST LIFT

SECOND LIFT

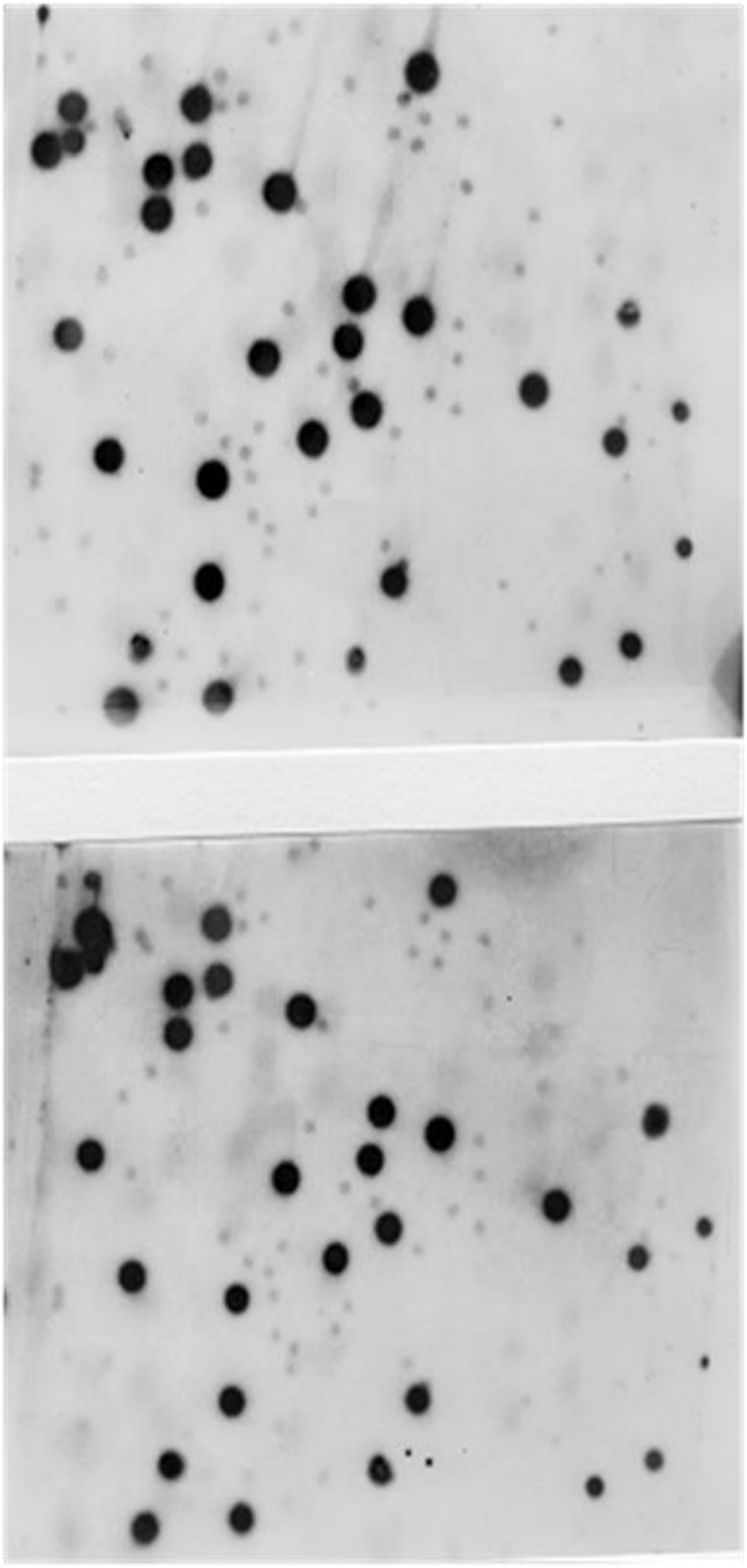

Figure: 2. The third round screen of a positive cDNA clone from the kidney *Xenopus laevis* cDNA library with the M-clone probe.

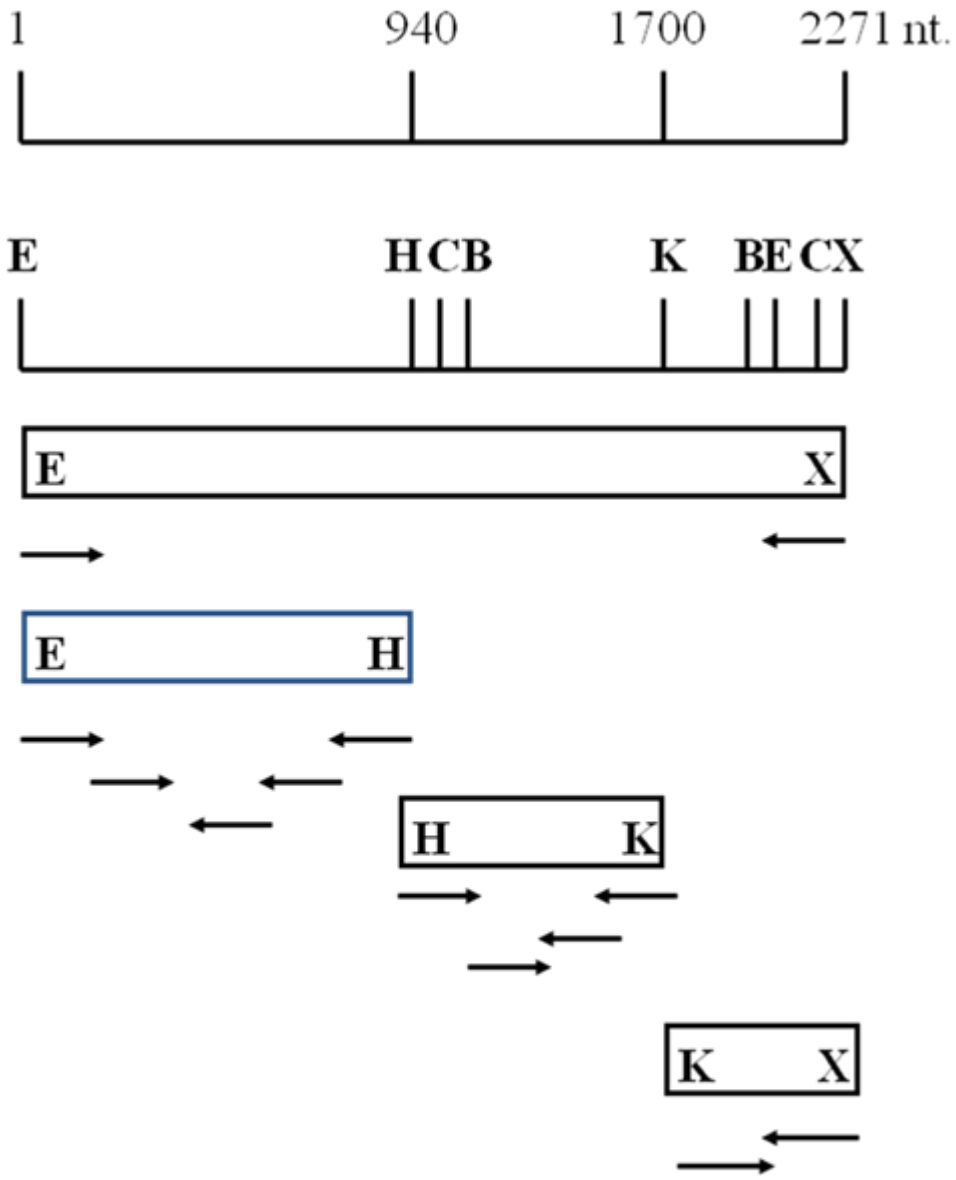

Figure:3. Sequencing strategy for x14k.

Sequencing was largely from the subclones indicated by the boxes, made in pBluescript (KS). The arrows indicate the direction and extent of sequencing. Sequencing was from the pBluescript (KS) T3 and T7 sequencing primers. B=BamHI, C=HincII, E=EcoRI, H=HindIII, K=KpnI, X=XhoI

Nucleic acid sequencing strategy

To determine the probe sequence, the 5' and 3'ends of the full-x14K were sequenced using the T3 and T7 priming sites within the vector. The sequence of several hundered base pairs at both the 5' and 3'ends showed that the probe sequence was not included in those non-coding regions. Open reading frames also were not found in those flanking regions. However, in order to establish a sequencing strategy, to the 2.271 kb full-x14K, EcoRI, HindIII and KpnI sites were used to subclone three fragments into pBluescript vector (KS). The restriction mapping of full-x14K showed that sequencing overlapping subcloned fragments of the insert would be lengthy due to the limited number of convenient unique restriction endonuclease sites. To avoid this the three subclones were sequenced inwards from T7 and T3 priming sites of the vector and thereafter five oligonucleotides primers (17-21 mer) were made using the sequence of subclones and used as sequencing primers.

These primers were from:-

(1) EcoRI/HindIII fragment.

HER: 5'-TCCTTGTACCATCCGTC-3'

HEL: 5'-AGTTACCAAGAGGCACG-3'

HB1: 5'-CTGTTCCTCTGTTCCGCTTG-3'

(2) HindIII/KpnI fragment.

HKA: 5'-CAGGAATGCTTCCGCTTAGTG-3'

HKB: 5'- AGTTCAGGCAAGGCATGATGG-3'

Full sequence analysis of x14K-gene

The x14K-cDNA was isolated by screening a kidney X. laevis cDNA library using the M-clone fragment (figure 1) as a probe and the insert was sequenced. The x14K-cDNA (figure 4) was 2271 nucleotide long and contained a stop codon at position 1392-1394. The sequence of x14K from 1062 to 1256 is identical to the probe sequence in clone M. This probe sequence is included in the translated region of x14K-gene. The sequences of the two primers that were designed from the M sequence: X5’-CCGCTTAGTGTTATGAGACG-3’andM5’GGATCAACCATCAT-3', and used in RT-PCR assays (probe paper) were also found in the x14K sequence, but I observed that the distance between the two primers in the x14K gene, which is 847nt (from 1131 to 1976nt) is larger than the distance between them in the M-clone. So the size of this fragment (847nt) has revealed the origin of band in RT-PCR analysis of retinoic acid inducibility described previously (probe paper), and strongly confirmed that x14K-gene is a retinoic acid responsive gene.

The 3’noncoding region of the cDNA is highly A + T rich and contains a typical polyadenylation signal (AATAAA starting at position 2225)

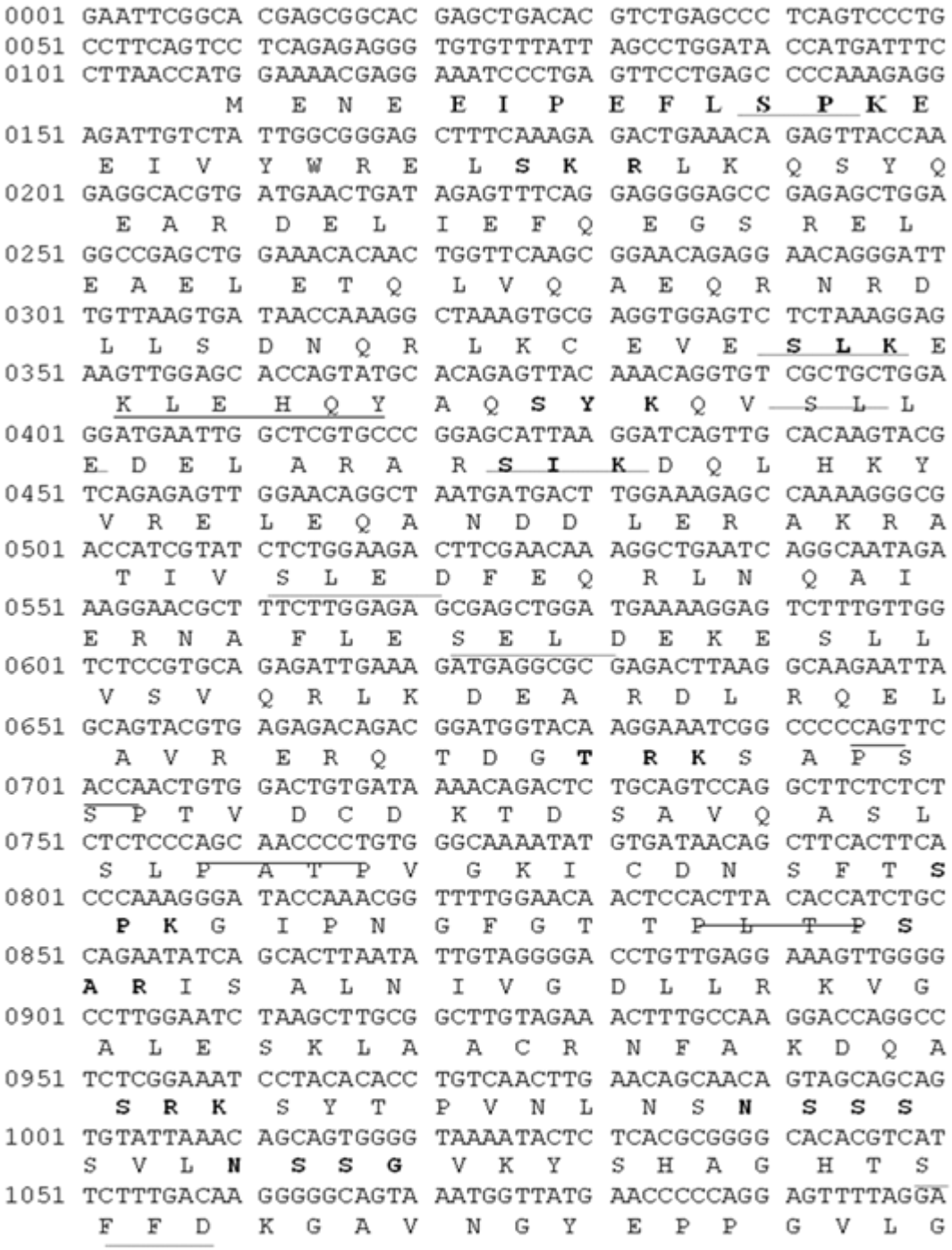

Figure:4. The nucleotide sequence and deduced amino acid sequence of x14K-gene. The nucleic acid sequence is numbered starting with the first nucleotide deduced within the 2271bp fragment. One long continous open reading frame (47kD) was obtained on translation and this is shown beneath the corresponding nucleotide.

```
1101 TCCCGGCCTC CGTCCCCCCC AGGAATGCTT CCGCTTAGTG TTATGAGACG
       S  R  P   P  S  P   P  G  M  L   P  L  S   V  M  R
1151 GAGACTTTCA TTAATTATTT ATATTTTTTT CCGGCATAAG AACAAAACTT
     R  R  L  S   L  I  I   Y  I  F   F  R  H  K   N  K  T
1201 GCTACGGTTT AAACCACTTT GCTGCCTCTC CTCTCCCCGG CAGACATGGG
     C  Y  G   L  N  H  F   A  A  S   P  L  P   G  R  H  G
1251 GTTAAACACT ATGTGATTGA GCTGCACTAC ATCATCTCCT CTGCTGCCTC
       V  K  H   Y  V  I   E  L  H  Y   I  I  S   S  A  A
1301 TCTGACACTT CATAAGGACT TGTCCTCCAG TTCTACCCGT GTATCGTGGT
     S  L  T  L   H  K  D   L  S  S   S  S  T  R   V  S  W
1351 TTGTTAATCG CCAACGAAAC CTCACCCACT GCTATGGTGA CTAATAATGC
     F  V  N   R  Q  R  N   L  T  H   C  Y  G   D  -  -
1401 TCCAGTATGG CATCTTCCCC CATCATGCCT TGCCTGAACT TGCCCGGGGG
1451 TAACCTGACG CAGCAGGTGA AAAGACAGAA GGAAGGGCCT TTTCTCACCT
1501 GCCAAGCACT ACTTTTTGGG GGTTCTGGCC ACCCCAGGAA TCATCCATCT
1551 ACGTTACAGG GTTAAAGCTG ACCTCATGTC GGCATTTGTT TATGGTTAAA
1601 CAGTGTTGTT GGAATATATT TTGCTGTGTT ACAAATAGGG CTTTAATCTG
1651 ACCTGCCGGG TCTCTTGGTA CCATTGTGGT CTTTCCAGCT CTATATTATA
1701 TATATATATA TATATTTGTG TATCTCCCCC CCCCTCCTCA TAAATACATG
1751 TAGATTCCAC CCAGTTTGAC TTAACAATGT CCAATTCTGT ATAATCCCAC
1801 TTCATTTCCA GCCCTACACC TCAATCCCTG ACTCTAAGTC TTGATTGGAT
1851 CCTTCCTGCA CATTACTTTT AAAGCACACT TAATTTTAAC CCCTAGTTAG
1901 CCTATATATA TATGTACATA TGTTTCTTTC CCCTTTCACC CTTGAACCTC
1951 AATGTTGGAA TTGTGATATG ATGGTTTACA TTTTCCTTTT TTTTTTTTGT
2001 TTTTGTTTTT ACTGTACATA GATTTGTAAA GATAATATTT TTGGCCTAAG
2051 ATATTTGTAC ATAACTTGGG CGCTGTAGCT ATATTTATTG AGAATTCTAT
2101 ACGGCATGTT TAAAGGAAGG CGATGCACAG TCCCACGGTT GCTGTCAACG
2151 CGCGCACACG AAAAAAACAA AAAACAAGAT GGACTCGGCG TCATGGTTGT
2201 GAAACGGTGA ATATTTTTAA AAAGAATAAA AATTTTCAAC GTGTAAAAAA
2251 AAAAAAAAAA AAAAAAAAAA A   ......
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Figure: 4 (continued)

followed by a poly (A) tail. The second in frame ATG (position 108-110) conformed to Kozak's rules for a translation initiation site and was presumed to be the translation initiation codon. The 5'untranslated region (107bp) which preceded the putative ATG did not contain an in-frame stop codon, which indicated that x14K-gene may not represent a full length cDNA. The amino acid sequence deduced from the single x14K-open reading frame (figure 4) predicts an 428 amino acid acidic protein with a calculated molecular mass of ~47kDa (figure 5). The N terminal region is rich in glutamine residues and is highly hydrophobic.

The region of the x14K-protein prior to residue 200 adopted stretches of α-helical conformation. This domain has the typical features of an α-helical coiled-coil structure. α-helical proteins with a coiled-coil structure contain a characteristic repeated heptad amino acid sequence (a, b, c, d, e, f, g) where positions a and d are generally occupied by small and apolar residues (Lupas et al., 1991). The residues 7-65 of x14K-protein contain clusters of heptad repeats. Within this domain all the residues at position a are glutamine. In contrast greater variability is observed at position d. These observations suggested that the x14K-protein will adopt a coiled-coil conformation. Since coiled coils form when two α-helical regions wrap around one another, x14K would be expected to form homodimeric or heterodimeric structures.

The 428 amino acid x14K-protein (figure 4) contains four consensus sequences for asparagine-linked N-glycosylation (Asn-Xa-Ser/Th-Xa).

It has consensus leucine zipper sequences (Leu-6aa-Leu-6aa-Leu-6aa-Leu) at residues 64-85 and 110-132. The N-terminal region of the amino acid sequence (at position 118) contains a potential site of tyrosine phosphorylation sequence in a consensus pattern: lysine-Xaaa-E-Xaa-Y.

Patschinsky et al. (1982) observed that the substrates of tyrosine protein kinases are generally characterized by a lysine or arginine seven residues to the N-terminal side of the phosphorylated tyrosine. An acidic residue, like asparagine and glutamine is often found at either three or four residues to the N-terminal side of the tyrosine. This character of tyrosine kinase phosphorylation site is identical to that found in the open reading frame of x14K-gene.

The x14K-protein sequence also contains ten protein kinase C phosphorylation sites in the following consensus patterns: six are in the form of serine-Xa-lysine, three in serine-Xa-arginine form and one in a threonine-Xa-arginine consensus pattern. It has four mitogen-activated protein (MAP) kinase sites in a consensus pattern: Pro-Xa-Ser/Thr-Pro.

From the protein kinase sites that are included in x14K-protein sequence is the casein kinase II (CK-2) consensus pattern: S/T-Xaa-D/E. Seven sites of CK-2 were found in the protein-sequence of x14K-gene. The phosphate acceptor site of CK-2 in these seven kinase sites was serine and the acidic terminal residue of the pattern was aspartate or glutamate.

To obtain further information regarding the possible function of x14K-gene, I compared its amino acid sequence with known protein sequences in the GenBank

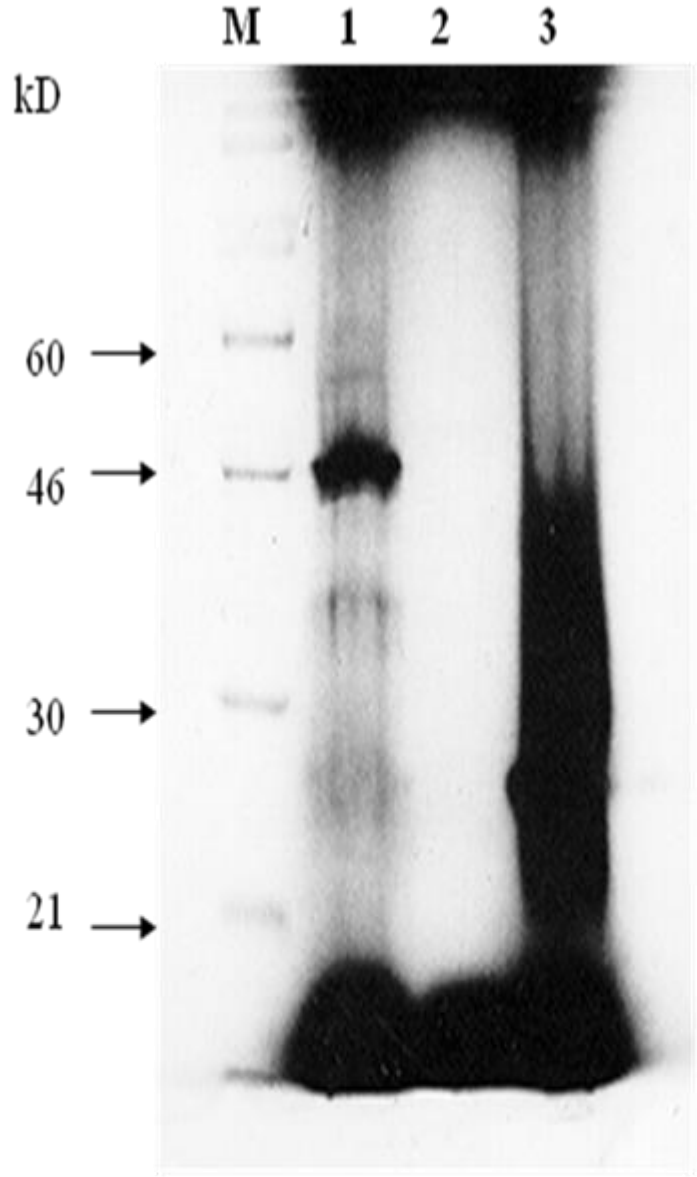

Figure: 5. *In vitro* transcription and translation of Pb RN3/x14K. The vector was transcribed with T3 RNA polymerase followed by translation (lane 1) of resultant RNA in a Rabbit reticulocyte lysate system. In lane 2, the RNA component of the translation system was replaced by water. Lane 3 represents the positive control of CAT mRNA. Lane M = [14C]-labelled rainbow proteins. The products of translation were analysed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

and protein information Resource. No homology was found with the COOH-terminal 139 amino acids of this polypeptide. But the NH_2-terminal 289 amino acids showed best homology of 84% to Xenopus laevis mitotic phosphoprotein43 (MP43; figure 6) . This latter protein has a consensus phosphorylation site for cyclin-dependent kinase II (cdc2) that appears to directly phosphorylate most of the mitotic phosphoproteins. The MP43 has two mitogen-activated protein (MAP) kinase sites similar to that found in x14K-protein. NH2-terminal region of x14K-gene has also homologies of 44-46% to two human epidermal growth factor substrates (AF-1P proteins highly similar to murine eps 15) and three human nucleoproteins (TPR). The data base program, revealed that x14K-protein sequence has similarities to a number of the myosin heavy chain (MHC) of human, rabbit, chicken, frog (X. laevis), Drosophila melanogaster, and Caenorhabditis elegans. There were also similarity between the N-terminal region of x14K-protein and regions of a number of skeletal and cardiac alpha tropomyosin, a Herpesvirus saimiri ORF73 homolog [Kaposi's sarcoma-associated herpes like virus] and several Saccharomyces cerevisiae proteins (like USO1 yeast proteins). These various proteins have also homologies with myosin family proteins (myosin, paramyosin, and tropomyosin)

and Saccharomyces cerevisiae protein.

Discussion

The analysis of the x14K-gene revealed a strong match with the Xenopus mitotic phosphoproteins 43 (MP43) (figure 5). The MP43 clone was partially sequenced by Stukenberg et al. (1997). They reported that the sequenced region of this protein does not bear significant similarity to

known proteins, but it has a consensus phosphorylation site of Cdc2 (also known as $p34^{cdc2}$) and two mitogen-activated protein (MAP) kinase sites. By using the screen for gel-mobility shifts, they found that the MP43 has a reduced mobility after incubation in a Xenopus mitotic extracts. When the MP43 in vitro translated protein was incubated with affinity-purified cyclin B-Cdc2, they observed that its mobility shift was the same as the mitotic extracts, suggesting that it was indeed a consequence of mitotic kinase activity. They also found that MP43 could be precipitated by MPM-2 immunoprecipitations (the anti-phosphoepitope monoclonal antibody MPM-2) and therefore can be called “true” MPM-2 antigens.

The homology between x14K-protein and the Xenopus MP43 is significant, and suggests that the X14k-protein may represent a novel Xenopus mitotic phosphoprotein.

As I mentioned, the x14K-protein has about 84% homology to MP43. A search for a conserved domain or motif among x14K-protein revealed different consensus phosphorylation sites for protein kinase C (PKC) and mitogen activated protein (MAP) kinase. Similar motifs are found in the Xenopus mitotic

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x14K    1 M E N E E I P E F L S P K E E I V Y W R E L S K R L K 27
          M * *   E       F   S   * E E I * Y W *   * *   *   K
MP43   41 M D D L E N N I F N S V E E E I L Y W K S V A M K Y K 67

x14K   28 Q S Y Q E A R D E L I E F Q E G S R E L E A E L E T Q 54
          Q     * E A *   E L   E F Q E   S R E   E A E L E   Q
MP43   68 Q C S E E A Q Q E L Q E F Q E A S R E Y E A E L E A Q 94

x14K   55 L V Q A E Q R N R D L L S D N Q R L K C E V E S L K E 81
          L * Q   E   R N R D L   S * N   R L *   E * * * * K E
MP43   95 L L Q T E G R N R D L F S E N N R L R M E L D A I K E 121

x14K   82 K L E H Q Y A Q S Y K Q V S L L E D E L A R A R S I K 108
          K   E   Q * * * * Y   Q * S   L E   * L * *   * * * *
MP43  122 K Y E E Q H S E N Y V Q I S T L E G D L S Q T K A V R 148

x14K  109 D Q L H K Y V R E L E Q A N D D L E R A K R A T I V S 135
          D Q L   K Y * R E L E Q A N D D L E R A K R A T I * S
MP43  149 D Q L Q K Y I R E L E Q A N D D L E R A K R A T I M S 175

x14K  136 L E D F E Q R L N Q A I E R N A F L E S E L D E K E S 162
          L E D F E Q R L N Q A I E R N A F L E * E L D E K E *
MP43  176 L E D F E Q R L N Q A I E R N A F L E N E L D E K E N 202

x14K  163 L L V S V Q R L K D E A R D L R Q E L A V R E R Q - - 187
          L L   S V Q R L K D E A R D L R Q E L A V * * * Q
MP43  203 L L E S V Q R L K D E A R D L R Q E L A V Q Q K Q - - 227

x14K  202 D C D K T D S A V Q A S L S L P A T P V G K I C D N S 228
          *   * *   D * * V Q A S * * * P * _ P * _____ *
MP43  238 E T E R M D T S V Q A S I A I P S A P L T P L S Q R G 264

x14K  229 F T S P K G 234 - - 235 I P N G F G T T P L T P S A R 249
            S                     *   * G *     T P L T P   A R
MP43  265 C A S T L T 270 - - 279 L D D G Y S G T P L T P C A R 293

x14K  250 I S A L N I V G D L L R K V G A L E S K L A A C R N F 276
          I S A L N I V G D L L R K V G A L E S K L A * C R N F
MP43  294 I S A L N I V G D L L R K V G A L E S K L A S C R N F 320

x14K  277 A K D Q A S R K S Y T - - - - - - - - - - - - - - - - 287
            * Q *     *     T
MP43  321 V H E Q S P N R P L T - - - - - - - - - - - - - - - - 331
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Figure: 6. Predicted amino acid sequence alignment of x14K and *Xenopus* mitotic phosphoprotein 43 (MP43) (Stukenberg et al., 1997). The cyclin-dependent kinase (Cdc2) sites (T-P-XX-R/K) are upperlined. The mitogen activated protein (MAP) kinase sites (P-X-S/T-P) are underlined. The identical residues are arranged.
*=conserved Residue; – = gap for maximum alignment

phosphoprotein MP43 and are found in many mitotic regulators including Cdc2, Cdc25, Cdc27 and Wee1 (Izumi and Maller 1993; Coleman et al. 1993).

The cell cycle is controlled by a family of cyclin-dependent kinases (CDKs), which are sequentially activated by the synthesis and destruction of their cyclin regulatory subunits (reviewed in King 1994). In metazoans, there appears to be temporal regulation of two families of mitotic cyclins, namely A-type and B-type cyclins. It is believed that the complex of Cdc2 and cyclin A is activated in G2 and early prophase, whereas complexes of both A-type and B-type cyclins with Cdc2 are activated during prophase and metaphase, and only B-type cyclins are present during late metaphase, after the A-type cyclins have been degraded (Draetta et al., 1989; Minshull et al., 1990; Hunt et al., 1992). Several Cdc2-related proteins that define the CDK family have been isolated (Hanks 1987; Hellmich et al., 1992; Meyerson et al., 1992).

CdK2, originally called Eg1 was first isolated in Xenopus laevis by differential screening of an egg cDNA library (Paris and Philippe 1990). The cdK2 promoter activity was identified throughout oogenesis and just after the midblastula transition (Paris et al., 1991). The sequence of M-clone, that has been recovered from retinoic acid treated stage 14 X. laevis RNA and used as a probe for isolating the full length of x14K-gene was compared to sequences in the GenBank data bases using the FASTA program. Only two genes have homology to this partial M-sequence, the X.laevis keratin gene type I and the X. laevis protein kinase (cdK2) gene promoter. The 3’end of M-clone sequence (figure 1) showed best homology of 81% to protein kinase (Cdk2). The remaining part of the sequence (195 nucleotides) which is identical to the open reading frame

of x14K-gene is related to various previously identified proteins (Hocevar et al., 1993) like: lamins (A, B, C) and Homo sapiens retinoic acid receptor alpha (RARα).

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مستخلص العربى:

ايجـاد الطـول الكامـل لجـين x14k المتـأثر بحمـض ال Retinoid المعـالج لأجنـة الضـفادع الأفريقية(Xenopus laevis).

استكمالا للورقة العلمية و الخاصة بإعداد مجسات متأثرة ب retinoids لمسح المكتبات الجينية.

تجـارب العلمـاء تؤكد تدخل حمض الretinoid وهو من مشتقات فيتـامين A فـي نمو الجهـاز العصبي المركزي و التمايز الخلوي المبكر في الفقاريات.

من حـزم حمـض النـووي المختـارة من طريقـة العرض التمـايزي لجـل ال polyacrylamide والمتوقع انها متأثرة عند المعالجة بالretinoid لآجنة الضفدعة الأفريقية, تم عزل حزمـة سمية بال- M وهذه القطعة من ال cDNAs رجحت أنها تحت سيطرة التحكم الزيادي لوجود حمض retinoid، حجم القطعة هو 279bp.

الحمض النووي لهذه الحزمة أستخلص من المرحله 14 لآجنة الضفدعة الأفريقية المعالجـة بـال-retinoid. قطع cDNAs للحزمة علمت عشوائياً اشعاعياً (randomly labelled) بوجود α(32-p)dGTP ومزيج من مختلف hexanucleotides حيث استعملت كمجس(probe) لمسح مكتبة

cDNA (cDNA library) المجهـزه مـن كليـة الضـفدعة الأفريقيـة فـي نواقـل ZAPII vectors.

مكتبة ال- cDNA فرشت في اطباق مناسبة والبقع (plaques) المختارة من الفيروسات الناقلة استرجعت لخيوط مزدوجـة (double strands) فـي نواقل ( ) Bluescript phagemid SK^-.

فبعـد التنقيـة وتقـدير كميـة cDNA المختـار, حللـت العينـة أوليـاً وذلـك بـالتخريط القطعي (restriction mapping) باستعمال الانزيمات القاطعة. فحددت ألاماكن(sites) المختلفـة للإنزيمـات القاطعـة(restriction enzyme) علـي الامتـداد الطـولى لـل-cDNA المختار من ألمكتبه بمجس حزمة M.

لآن المجس(probe) الذي استعمل في مسح المكتبة الجينية المذكورة استخلص من مرحله 14 المعالجة لأجنة الضفدعة الأفريقية ولان cDNA المختار بعد المسح هو من مكتبة الcDNA لكلية(kidney) الضفدعة الأفريقية. من هنا جاءت تسمية الجين المتحصل عليه وهو جين x14K والمتأثر بحمض ال- retinoid .

عينات نتائج القطع بالانزيمات المختلفة مررت بطريقة ال- electrophoresing خلال ال agarose جل. منها حددعدد اماكن كل إنزيم قاطع وحجم المسافات البينية لها علي امتداد جين x14K المكتشف. قطع cDNA لكل إنزيمين تم حفظها (subcloned) داخل نواقل (vectors) مناسبة.

التسلسل القاعدي (sequencing) لمنطقتي نهايتي (5'end & 3'end) الطول الُكلي لجين x14K تم تحديدها وذلك باستعمال الأماكن القاعدية لكل من priming T3 & T7 المتوفرة ضمن الناقل (SK) Bluescript phagemid للمكتبة وال primer الخماسي المصمم من التسلسل القاعدي لقطع(fragments) الانزيمات المحفوظه(subclones) تدريجياً.

حجم جين x14K حدد بعدد 2271 قاعدي (mucleotides) طولاً ويحتوي علي شفرة (codon) توقيف عند موضع 1394-1392. التسلس القاعدي لجين x14K من 1062 الي 1256 هو للتسلسل القاعدي لمجس القطعة M (cloned M) والمسترجعه من أجنة الضفدة الأفريقية المعالجة بحمض ال retinoid. هذه القطعة (المجس) وتجارب أخرى طبقت علي جين x14K تؤكد بقوة أن الجين (x14K)المكتشف هو تحت تحكم وسيطرة حمض ال retinoid ومستقبلاته (retinoic acid receptors).

منطقة cDNA للنهاية الثالثة الغير مشفرة (3'non coding) من ألجين وجدت أنها غنية بقواعد A+T وتحتوي علي ترتيب نموذجي من تعدد قواعد A (Polyadenylation signal) والبادئة عند الموضع 2225 قاعدي حيث تذيل بال- Poly(A)tail.

ترتيب الحمض الاميني المتحصل عليه من المنطقة المشفرة قدر ِب428 حمض أميني لبروتين حامضي بقراءة وزنية جزيئية تعادل 47KDa~.

وفي ورقة علمية قادمة قمت بتحديد موضع هذا ألجين(in situ hybridization) في الجهاز العصبي المبكرفى اجنة الضفدعة الأفريقية كما أجريت تجارب حقنه (ألجين) في أجنة الضفدعة ورأيت مدى تأثيره علي التركيبة العضوية لأجنة ضفدعة ال- *Xenopus laevis*.