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MOLECULAR CLONING OF CAMK2A GENE

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Abstract: *The role of calcium/calmodulin-dependent protein kinase II (CaMKIIα) as a signaling molecule inside neurons could influence function of the brain in learning and memory. Calcium signaling is crucial for several aspects of plasticity at glutamatergic synapses. CaMKIIα drives synaptic insertion of new receptor subunits and phosphorylates receptor subunits directly to enhance channel conductance. These processes are widely believed to play critical roles in the long-term regulation of synaptic transmission at the cellular level, and in learning and memory. The molecular cloning of the gene in mouse and rat could amplify and grow it. Sequencing results from the forward primer showed that the construct was present and it matched with the initial portion of the untranslated region, 3'UTR of CAMK2A gene of mouse. The reverse primer gave no sequence, probably because the Kanamycin portion got deleted from the construct. It was also seen that the end part of the sequence from forward primer had a portion of WPRE (Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element). This also validated the fact that Kanamycin portion got deleted. The sequencing results from the WPRE reverse primer had a major portion of the WPRE gene and then had a small portion of the CAMK2A 3'UTR. But this portion was from a middle portion of the UTR. This showed that the construct did not have the entire 3'UTR in it. The gel did not give any band anywhere after all three* polymerase chain reaction*s, which showed that it did not get amplified. The translation regulation of the protein encoded by the gene could prove the long-term regulation of potentiation and depression and thus learning and memory.*

Keywords: *Calcium/calmodulin-dependent protein kinase II, synaptic transmission, synaptic plasticity, memory, cloning, polymerase chain reaction, cDNA.*

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1. INTRODUCTION

Since the time scientists recognized that biochemical events inside neurons could influence the function of the brain, there have been people looking for “memory molecules.” This elusive ion, small molecule, protein, or nucleic acid would be the keystone on which memory was built; understanding the memory molecule would allow us to unlock the mysteries of cognition. Models of how this molecule could work abounded, but the idea of a memory molecule as a kinase/phosphatase-based molecular switch emerged as an important contender [1]. The discovery that there actually was an abundant neuronal protein kinase that had switch-like properties [2] was met with great enthusiasm, and the role of calcium/calmodulin-dependent protein kinase II (CaMKIIα) in learning and memory has been studied intensively.

CAMK2A (Calcium/calmodulin dependent protein kinase) encodes for the protein CAMKIIα. CaMKIIα is a ubiquitous, high-abundance signaling molecule. It is a serine/threonine kinase with a broad range of substrates. CaMKIIα is found in most tissues, but it is present in especially high concentrations in neurons, in which it may be up to 2% of total protein in some brain regions [3]. CaMKIIs are encoded by four genes in mammals (α, β, γ and δ), each of which is transcribed and processed into a variety of alternatively spliced mRNA products.

CAMKIIα mediates diverse physiological responses to increases of intracellular Ca 2+ concentrations by virtue of its activation by Ca 2+ /calmodulin and autophosphorylation. Calcium signaling is crucial for several aspects of plasticity at glutamatergic synapses. CAMK2A is most highly expressed in neurons, where it modulates most aspects of neuronal function, including gene expression, neurotransmitter synthesis and exocytosis, neurotransmitter receptor and ion-channel functions, cytoskeletal interactions and morphology, and various signalling pathways. CaMKIIα drives synaptic insertion of new receptor subunits and phosphorylates receptor subunits directly to enhance channel conductance. These processes are widely believed to play critical roles in the long-term regulation of synaptic transmission (long-term potentiation and depression) at the cellular level, and in complex animal behaviour, such as learning and memory [4]. The synaptic specificity of many of these changes indicates that localization of CaMKIIα actions to specific synapses is critical to its function in vivo.

The evidence that CaMKIIα is involved in memory formation is quite convincing and comes from multiple vertebrate [5, 6] (reviewed mouse and rat studies) and invertebrate [7, 8] species. Key experiments performed in mice have shown that the disruption of CAMK2A impairs long-term potentiation [9, 10] and long-term memory [9]. Moreover, mutations in genes involved in dendritic mRNA targeting or translation have been linked to several human neurological disorders, including the most common cause of inherited mental retardation fragile x syndrome, consistent with a role for dendritically localized protein synthesis in the regulation of synaptic morphogenesis and plasticity [11].

The 3′-UTR in the mRNA encodingCaMKIIα contains two cytoplasmic polyadenylation elements (CPEs). These CPEs interact with a CPE-bindingprotein (CPEB) that is present in hippocampal dendritesand enriched in post synaptic densities. Binding of CPEB to the 3′-UTR ofCaM-KII mRNA promotes its polyadenylation, thereby enhancing translation [12].In general 3' UTR is a target of micro RNA mediated translational regulation. Thus it is important to study the translation regulation of the protein encoded by the gene. To do this, we need to make the clone of the 3' UTR of this gene with some reporter like luciferase. The aim of this project is to clone the gene into pGL3 vector which has luciferase gene as the reporter.

Molecular cloning

Molecular cloning is the process by which a DNA molecule of interest is taken out of a source DNA and is joined with the DNA of a vector, which is transformed into a host organism, where they are replicated. The DNA of interest is either cut out of the source DNA by cutting with restriction enzymes or copying it using polymerase chain reaction (PCR). The plasmid is a small, circular piece of DNA that is replicated within the host and exists separately from the host's chromosomal or genomic DNA. The plasmid vector is made into a linear form by cutting with restriction enzyme or by using PCR. By joining the gene of interest with the plasmid, the new recombinant plasmid is replicated by the host. This helps to generate large amounts of the gene of interest easily and can also provide the control to direct transcription or translation.

Traditional cloning

Traditional cloning is the method in which restriction enzymes are used to generate DNA fragments from the source and vector so as to create complementary end sequences that

can be joined together with a DNA ligase. This is done by cutting with two unique restriction enzymes that flank the DNA sequence and are also present at the site of insertion of the vector (multiple cloning sites). By using two restriction endonucleases (RE), two non-compatible ends are made and so the insert will be cloned directionally. This decreases the chance for the vector to be re-ligated, and also to get inserted in the correct orientation, in which case, the clones need to be screened to check for the correct orientation.

PCR cloning

PCR cloning is the method where the DNA of interest and vector is amplified by PCR and ligated together, without the use of REs. It allows for the cloning of DNA fragments that are not available in large amounts. The PCR reaction is used to amplify the DNA of interest and is the joined to the vector. PCR cloning generally uses Taq DNA polymerase to amplify the DNA. High fidelity polymerases are also used to amplify the DNA.

Preparation of DNA

This involves the preparation of vector backbone and the insert. When starting with DNA, restriction enzyme digestion or PCR is performed. When starting with RNA, cDNA is synthesised using a reverse transcriptase. This can be used as a template for any of the cloning methods listed previously. Depending on which workflow is being followed, the resulting DNA may require a clean-up step. This can be performed using a spin column or by gel extraction.

Restriction enzyme digestion (for traditional cloning)

Restriction enzyme sites that are unique to both the insert and vector should be chosen. Unidirectional cloning is achieved using two different restriction enzymes, each with unique recognition sites at an end of the insert.

Vector and insert ligation

The formation of a phosphodiester bond between the 3´ hydroxyl and 5´ phosphate of adjacent DNA residues proceeds in three steps: Initially, the ligase is self-adenylated by reaction with free ATP. Next, the adenyl group is transferred to the 5´ phosphorylated end of the “donor” strand. Lastly, the formation of the phosphodiester bond proceeds after reaction of the adenylated donor end with the adjacent 3´ hydroxyl acceptor and the release of AMP. In living organisms, DNA ligases are essential enzymes with critical roles in DNA replication and repair.

Transformation

Transformation is the process by which an organism acquires exogenous DNA. Artificial transformation encompasses a wide array of methods for inducing uptake of exogenous DNA. In cloning protocols, artificial transformation is used to introduce recombinant DNA into host bacteria. The most common method of artificial transformation of bacteria involves use of divalent cations (e.g. calcium chloride) to increase the permeability of the bacterium's membrane, making them chemically competent, and thereby increasing the likelihood of DNA acquisition. With a newly-compromised cell membrane, the transforming DNA is free to pass into the cytosol of the bacterium.

DNA analysis

Agarose or polyacrylamide-gel electrophoresis is the standard method used for separation, identification and purification of DNA fragments. DNA is visualized on a gel after soaking or pre-impregnating the gel with ethidium bromide, a DNA intercalating agent that fluoresces under UV illumination. Using the marker or ladder as a reference, it is possible to determine the size and relative quantity of the DNA of interest. The original DNA markers were made of genomic DNAs digested with a restriction enzyme to exhibit a banding pattern of known fragment sizes. Later, markers were made of fragments with evenly-spaced sizes and the resulting banding pattern resembles a ladder. The bands are visible under UV illumination and the bands of the marker/ladder are not visible under normal lighting conditions. To track the progress of the gel as it runs, the marker contains a dye or combination of dyes that identify the leading edge of well contents, also called the dye front.

Steps in PCR

Initialization isonly required for DNA polymerases that require heat activation by hot-start PCR[13]. This consists of heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermostable polymerases are used), which is held for 1–9 minutes. Denaturationis the first regular cycling event (Fig. 1) and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules. In annealing, the reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. This temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the

hybridization to be specific, i.e., the primer should only bind to a perfectly complementary part of the template. If the temperature is too low, the primer could bind imperfectly. If it is too high, the primer might not bind. Typically the annealing temperature is about 3–5 °C below the melting temperature of the primers used. Stable DNA–DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation. The temperature inextension/elongationstep depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to amplify. As a rule-of-thumb, at its optimum temperature, the DNA polymerase polymerizes a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment. Final elongationstep is occasionally performed at a temperature of 70–74 °C (this is the temperature needed for optimal activity for most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended. Final hold step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

qPCR

The method of choice for nucleic acid (DNA, RNA) quantification in all areas of molecular biology is real-time PCR or quantitative PCR (qPCR). The method is so-called because the amplification of DNA with a PCR is monitored in real time (qPCR cyclers constantly scan qPCR plates). It is, in contrast to the conventional PCR, quantitative, meaning that it enables us to determine the exact concentration (relative or absolute) of the amplified DNA in the sample. Conversely, in conventional PCR we can see the result of amplification only after the PCR is completed (end-point detection).PCR is a method where an enzyme (thermostable DNA polymerase) amplifies a short specific part of the template DNA (amplicon) in cycles. In

every cycle the number of short specific sections of DNA is doubled, leading to an exponential amplification of targets.

In qPCR, exactly the same procedure happens but with two major differences: first the amplified DNA is fluorescently labelled (usually with cyanine based fluorescent dyes) and second, the amount of the fluorescence released during amplification is directly proportional to the amount of amplified DNA. Fluorescence is monitored during the whole PCR process (along all 30 to 45 cycles). The higher the initial number of DNA molecules in the sample, the faster the fluorescence will increase during the PCR cycles (first and second images of Fig. 1). In other words, if a sample contains more targets the fluorescence will be detected in earlier cycles. The cycle in which fluorescence can be detected is termed quantitation cycle (Cq) and is the basic result of qPCR. Lower Cq values mean higher initial copy numbers of the target. This is the basic principle of quantitative approach that real-time PCR offers.

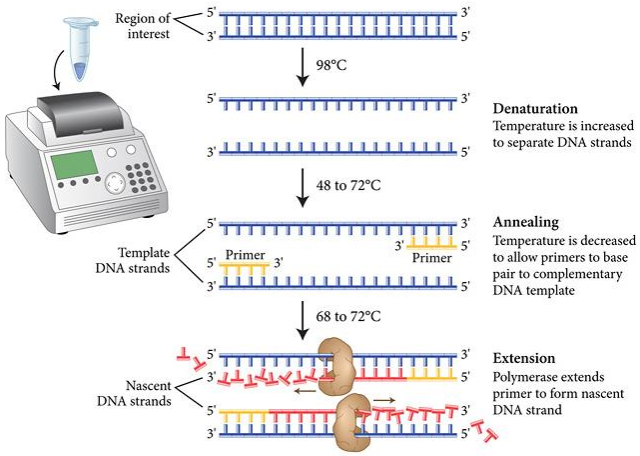

Fig. 1. Steps in PCR

2. MATERIALS AND METHODS

Isolation of construct from FIV eGFP vector and incorporation to pGL3 vector

A construct which had the CAMK2A 3'UTR incorporated in the FIV eGFP (Feline Immunodeficiency Virus, enhanced Green Fluorescent Protein) vector (Fig. 2) was used. Construct was tried to be isolated and incorporate it to the pGL3 vector (Fig. 3).3' UTR sequence of CAMK2A gene of mouse is shown in Fig. 4. For this we sequenced the construct to know about the restriction enzyme sites with which to cut out the gene of interest and also to find if the CAMK2A was that of mouse or rat using forward primer, eGFP and reverse primer, Kanamycin. The construct was given for sequencing again with Forward primer as

eGFP and M13 as reverse primer.

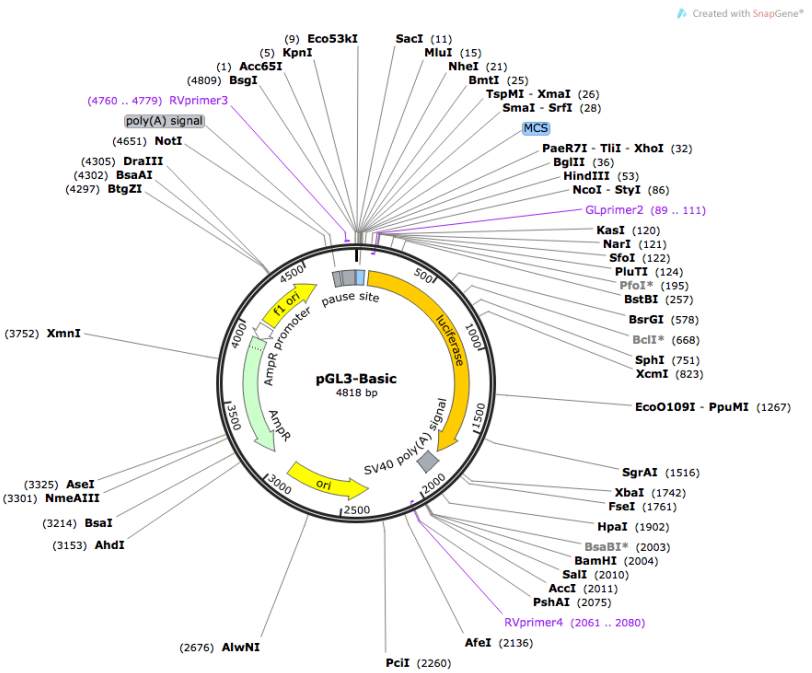

Fig. 2. FIV eGFP vector backbone circle map

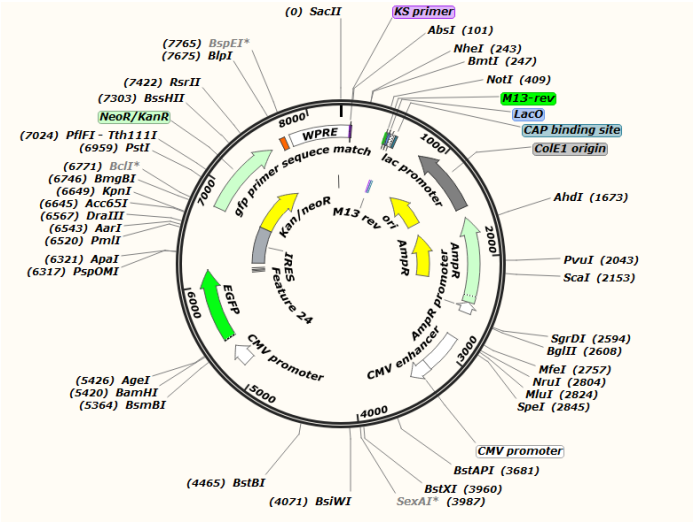

Fig. 3. pGL3 vector circle map

AGGACCAGGCCAGGGTCCCTGCGTCCTTGCTTCGCAGAGATCCGCTCTTTGTCCGTGGAATGTGGCT
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CGCGTCACGAAAACCTGCTTGTTCACAGAAGTCGCCACGACATCACAGTGAACAGCCAGCTCTCCCC
AGCTCCGTTGCCCAAGCTCTTCCTGCCAGTGGGGACCTTCTTCCGGCTTAAGTACCCAGGGTGCTGG
CCCCAGGAACCCCCACCCCCTACCCACTGTTGTTGGCCTAGCCTAGCTTTAGCTATAGATGGGGCCTC
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GATTTTGCGTTAACGGACATCTATGTGATAACTAGACCTCTCAAGTTTACTGTAAAGAAGAGTCGGA
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TGGGAGCAGAAGTGGAAGGGATGTTTAATCCAGAACTTTCTGGTATTCCCCTTTCGCCTACATGTGA
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GGGCCCTTGTCCTGCCCCAGAACCAGGCTTCTTCATAAGGCTCAGCAAACCCATTGTCCCCAGCCACT
CCCCAGATAAAGGTAAAGGAGGGTGTGGCCTTTACCAGGGGACACTGCAATCTCCATGCAGGATCT
ACAATCCCTTCTTCAAGCCTCAGTTTCTCCATCAATGTTCCTACCCAGACTGATGGAGGGTCAGAGTA
AAAGATGTCACAAGCACCCACCACCTCTGAGAGCTTGTTGGGTTTGTCACTGGCTGGCCCTCTTATG
CACCAGGTCTGGCCAACCCCCACCCTTTCCTCTGTGTGCCCCTCATTTTACTATTTGGTGCCAGTCCGT
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ATCCGGGTTTTGTGTAGCTTTGGGTAAATCCTTTGCCCTCTTCAGGCTTTAGTTTCGCCAACCAGAAG
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GCAGCCAACTAATGAAGACCTTGGGACACTACGATGGCCCCAAGGTGACAGGCAGGGGAACAGGC
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CTGGTTTATTCTTGGCGTTTTCAGTGGAATAAATAGCTACAAATTTATGTGAGTCCGTGTCTTCCTGA
ATTGGTCAAGGCACAGAGCCCCAGGAACTGGCATTTTGCTTTGGCTTGTTTTTTGGGTTTTTTGTTTG
TTTGTTTGTTTTTGTTTGTTTCAAATCTCCCCTGTTGCAAAATAAAAGTCCTGGTCCTATGGATTGGAA

Fig. 4. 3' UTR sequence of CAMK2A gene of mouse

cDNA synthesis and amplification of the gene of interest

cDNA was synthesised from RNA of rat. Using primers specific to the CAMK2A (with flanking RE sites), gradient PCR was done for amplifying the gene of interest. Agarose gel

electrophoresis was done to verify this. PCR was again done with a single temperature (56 C). Agarose gel electrophoresis was also done.

Primers used for PCR are; forward primer: atctagaTGAAGGACCAGGCCAG and reverse primer: agtcgacAAATTTGTAGCTATTTATTCCACTG (sequence in small letters correspond to the RE; XbaI for forward primer and SalI for reverse).

qPCR was done for the cDNA to check if the gene was present in sufficient amount with primers specific to the gene of interest. Gradient PCR was done again using a Phusion high fidelity DNA polymerase followed by electrophoresis.

For cDNA synthesis, the RNA was denatured by keeping a mixture of RNA, primer, dNTPs and water at 65^{o} C for 5 minutes. It was kept on ice for about 1 minute. Annealing was done by adding Oligo(dT)20 and Random Hexamers by keeping at 25^{o} C for 10 minutes. cDNA synthesis mix consist of10X RT buffer-2 µl;25mM MgCl2-4 µl;0.1M DTT-2 µl;RnaseOUT-1µl and superscript III RT-1µl.The synthesis was done at 50^{o} C for 50 minutes. The reaction was terminated by keeping at 85^{o} C for 5 minutes, chilled on ice, centrifuged briefly and then RNAase H (1 µl) was added and kept at 37^{o} C for 20 minutes to remove the RNA, and the cDNA was stored at 20^{o} C

3. RESULTS AND DISCUSSION

Isolation of construct from FIV eGFP vector and incorporation to pGL3 vector

Sequencing results from the forward primer showed that the construct was present and it matched with the initial portion of the 3'UTR of CAMK2A of mouse (Fig. 5). The reverse primer gave no sequence, probably because the Kanamycin portion got deleted from the construct. It was also seen that the end part of the sequence from forward primer had a portion of WPRE. This also validated the fact that Kanamycin portion got deleted.

CCCCCCACCCGGCTCTCCTCGGCATGGACGAGCTGTACAAGAAGCTTAGCCATGGCTTCCCGCCGGA
GGTGGAGGAGCAGGATGATGGCACGCTGCCCATGTCTTGTGCCCAGGAGAGCGGGATGGACCGTC
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TTTACCCATTCCAGAGGTCTTTTTTTGGGGGGTGTCCCTTCTGGGGAGGTTGCTTTCTGGAGGTGCCT
CACCCCCAGGGAACATGGTTCTCAATTATGGTCCAGGTCAGCTGACAAAGATTTCTTCCGAGTCCGC
ATGACTACCACTGAGATCAGTCAGAAGCTATCTGAGGTTATCTGACTGGACACTCTCTGGAACTGTG
AC

Fig. 5. Sequencing result by using eGFP forward primer

The sequencing results from the WPRE reverse primer had a major portion of the WPRE gene and then had a small portion of the CAMK2A 3'UTR. But this portion was from a middle portion of the UTR. This showed that the construct did not have the entire 3'UTR in it.

cDNA synthesis and amplification of the gene of interest

The gel did not give any band anywhere after all three PCRs, which showed that it did not get amplified. Gel image after running gel electrophoresis of gradient PCR product is shown in Fig. 6 and that after amplification is provided in Fig. 7.

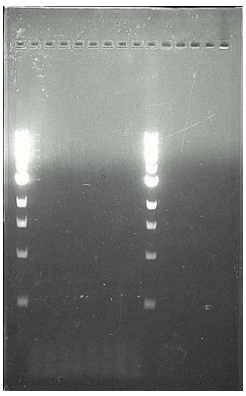

Fig. 6. Gel image after running gel electrophoresis of gradient PCR product

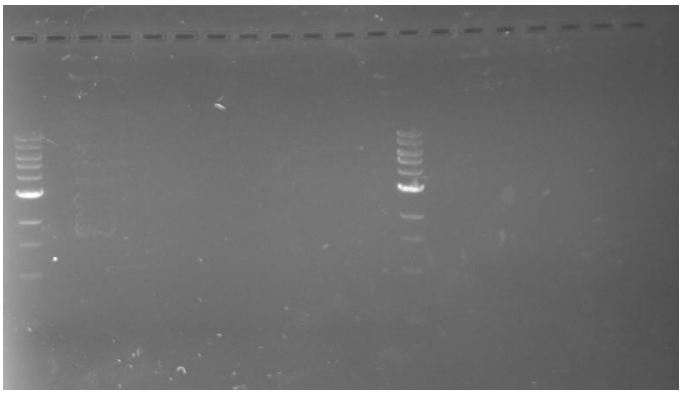

Fig. 7. Gel image after running gel electrophoresis of amplified gradient PCR product

4. CONCLUSIONS

None of the previous studies have shown the details of all primers used in their studies and

hence could not depend on reliability of those results reported. After making the clone and amplifying it by incorporating it into *E.coli* and growing them, it could be used to study the translational regulation of CAMK2A gene by using luciferase as a reporter. This could help in the better knowledge of the role of it in memory and learning and also probably help us understand its role in the fragile x syndrome and also help in the research towards a cure for it.

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REFERENCES

1. Lisman, J.E. (1985) A mechanism for memory storage insensitive to molecular turnover: a bistable autophosphorylating kinase. *Proceedings of the National Academy of Sciences*, 82(9): 3055-3057.
2. Miller, S.G. and Kennedy, M.B. (1986) Regulation of brain Type II Ca2+ calmodulin-dependent protein kinase by auto phosphorylation: A Ca2+-triggered molecular switch. *Cell*, 44(6): 861-870.
3. Erondu, N.E. and Kennedy, M.B. (1985) Regional distribution of type II Ca2+/calmodulin-dependent protein kinase in rat brain. *The Journal of Neuroscience*, 5(12): 3270-3277.
4. Lisman, J., Schulman, H. and Cline, H. (2002) The molecular basis of CaMKII function in synaptic and behavioural memory. *Nature Reviews Neuroscience*,3(3): 175-190.
5. Zhao, W., Lawen, A. and Ng, K.T. (1999) Changes in phosphorylation of Ca2+/calmodulin-dependent protein kinase II (CaMKII) in processing of short-term and long-term memories after passive avoidance learning. *J. Neurosci. Res.*, 55: 557-568.
6. Gleason, M.R., Higashijima, S.I., Dallman, J., Liu, K., Mandel, G. and Fetcho, J.R. (2003) Translocation of CaM kinase II to synaptic sites in vivo. *Nature Neuroscience*, 6(3): 217-218.

7. Fisher, A.J., Thompson, T.B., Thoden, J.B., Baldwin, T.O. and Rayment, I. (1996) The 1.5-Å resolution crystal structure of bacterial luciferase in low salt conditions. *Journal of Biological Chemistry*, 271(36): 21956-21968.
8. Wilson,E. (1999)What's That Stuff?*Chemical and Engineering News,*77(3): 65.
9. Miller,S., Yasuda, M., Coats, J.K., Jones, Y., Martone, M.E. and Mayford, M. (2002) DisruptionofdendritictranslationofCaMKIIalphaimpairs stabilizationofsynapticplasticityandmemoryconsolidation. *Neuron*, 36(3): 507-519.
10. An, J.J., Gharami, K., Liao, G.Y., Woo, N.H., Lau, A.G., Vanevski, F., Torre, E.R., Jones, K.R., Feng, Y., Lu, B. and Xu, B. (2008). Distinct role of long 3' UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. *Cell*, 134(1): 175-187.
11. Liu-Yesucevitz, L., Bassell, G.J., Gitler, A.D., Hart, A.C., Klann, E., Richter, J.D., Warren, S.T. and Wolozin, B. (2011) Local RNA translation at the synapse and in disease. *The Journal of Neuroscience*, 31(45): 16086-16093.
12. Wu, L., Wells, D., Tay, J., Mendis, D., Abbott, M.A., Barnitt, A., Quinlan, E., Heynen, A., Fallon, J.R., Richter, J.D. (1998) CPEB-mediated cytoplasmic polyadenylation and the regulation of experience-dependent translation of α-CaMKII mRNA at synapses. *Neuron*, 21:1129-1139.
13. Knivett,V. (2009)Lighting the way. *Electronic Engineering Times*. http://www.eetimes.com