行政院國家科學委員會專題研究計畫　成果報告

缺血後再灌流細胞傷害之酪氨酸激妹酶調控機制(I)大鼠腎臟模式

計畫類別：個別型計畫
計畫編號：NSC91-2314-B-002-408-
執行期間：91 年 08 月 01 日至 92 年 09 月 30 日
執行單位：國立臺灣大學醫學院醫學系

計畫主持人：賴明坤

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 92 年 12 月 31 日

行政院國家科學委員會補助專題研究計畫

成果報告

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共同主持人：
計畫參與人員： 王國忠

一、中文摘要

關鍵詞：細胞凋亡，缺血／再灌流，訊息傳遞，蛋白質酪胺酸激脢

腎臟移植後延遲發揮功能是因缺血後再灌流造成腎小管傷害發生細胞凋亡現象所致。這傷害可能因再灌流血液中富含氧氣而釋放自由基有關。前人研究氧化狀態誘發腎絲球細胞凋亡與細胞內訊息傳遞路徑，特別是酪胺酸激脢(PTK) – c-Jun/AP1 有關。本研究室近來發現因缺血後再灌流造成細胞凋亡可以使用自由基清除劑減少腎小管傷害。以水溶性碳六十前處理離體腎或活體腎均明顯減低腎傷害與細胞凋亡現象並且調控數種凋亡基因。然而目前對氧化自由基傷害及隨後復原中酪胺酸激脢訊息傳遞路徑了解不多。 本研究目標是分析缺血後再灌流中訊息傳遞的路徑並提供保存器官功能之機制以利腎臟移植。

本研究以活體大白鼠兩側腎動脈遭阻斷四十五分鐘後再遭受不同時段的血液灌流造成急性腎衰竭作為研究模式，探討腎臟遭受缺血後再灌流之訊息傳遞的情形。為涵蓋不同之傷害及復原階段變化，缺血再灌流腎臟在處理後 0，4，24，72，240 小時摘下分離純化RNA。這些RNA以一組de-generated DNA 引子PCR增幅一段 PTK 基因族群之保守區間DNA。長度 170 base pair 的PCR產物和 TA-vector 接合後以便進行 DNA 定序工作，經ampicilin抗生素篩檢正確接合質體。逢機挑選出菌株以DNA定序然後再以Dig作成探針。 排除相同的基因之後所有探針混合對先前選殖的PTK library 進行雜交。沒有黏合的菌株再逢機挑出菌株作DNA定序然後以Dig 作成探針，這些排除相同之Dig探針又和相同的colony lift 膜進行雜交以排除相同的基因。這種排除雜交法重複三到四次便可大量的篩檢每一個PTK基因。本研究將使用可容 4000 colonies 的colony lift 膜以達到選中每一個PTK 基因的可能，由於排除雜交法實際上涵蓋所有在膜上的每一個菌株，其測出篩檢的敏感度會在 0.025% 左右。每一株個別的PTK 基因以 Dot blot 方式做成一張含有全部篩檢到PTK基因的分析膜，再以不同缺血處理之腎臟RNA反轉錄做成一組特異性cDNA Dig探針和此分析膜作雜交即可得缺血處理間不同PTK 訊息傳遞之圖譜結果。比較處理間圖譜可望得到訊息傳遞之路徑基因調控。

本研究成果可解釋缺血後再灌流傷害中有關 PTK 訊息傳遞的機制及移植時可能的

腎臟功能保存方法，或減低缺血時細胞傷害。另外由本研究衍生的 PTKs 基因 library 膜可提供其他用大白鼠為模式的研究 PTK 者使用。

Abstract:

Keywords：Apoptosis, ischemia/reperfusion, signal transduction, protein tyrosine kinase

Apoptosis is induced after ischemia/reperfusion injury, which diminishes renal function after transplantation. The damage might be related to the release of free radicals in the oxygen rich reperfusion. Oxidant-induced apoptosis involved intracellular signaling pathways, especially the tyrosine kinase-c-Jun/AP1 pathway in the glomerular. In our laboratory, we found ischemia/reperfusion apoptosis injury can be reduced by free radical scanvenger fullerenes in renal tubular cells. The pretreatment of water-soluble fullerenes regulate several apoptosis genes, however, the knowledge of protein tyrosine kinase pathway regulated by oxidative stress and the subsequent recovery were limited. The aims of this project are to elucidate the signal transduction pathway in ischemia/reperfusion and may provide some protecting mechanisms to preserve the organ function during renal transplantation.

Wistar rats are used to induce ischemic renal failure. One of the renal arteries will be interrupted by hemoclip for 45 minutes and removed to reperfuse the kidney, the other kidney in the same rat will be used as control. The ischemia/reperfused kidneys will be harvested after 0, 4, 24, 72, and 240 hours after the treatment to cover the various stages of damages and recoveries. The RNAs will be isolated from the kidneys and a set of de-generated protein tyrosine kinase (PTK) pcr primers are used to amplify a conserved region in the PTK gene family. The proposed 170 base pair pcr products will be cloned into TA-vector for DNA sequecing. Randomly picked colonies will be sequenced and Dig-labeled. The identified genes will be pooled and hybridized the previous cloned PTK library. The non-hybridized colonies will again randomly be picked, sequenced, and Dig-labeled to hybridize the same colony lift membrane. The elimination hybridization cycle will repeat for 3 to 4 times to identify every PTK cloned. The colony lift membrane about 4000 colonies will be used in order to cover most PTK species in rat. The sensitivity of this screening and the subsequent analysis is estimated arround 0.025% in the mRNAs

population. Dot blot of every identified cloned will be used to hybridize Dig-labeled cDNA probe from the ischemic kidney mRNAs.

The results of this project can provide useful informations of PTKs signal transduction and possible therapeutic interventions for renal transplantation or ischemic damages. Furthermore, the dot blot membrane of rat PTKs can be shared to other researcher using rat as experimental model.

Objective:

Background

Kidney transplantation is one of the most routine solid organ replacement therapy at present time. Many evidences show that a good prognosis rely on several factors including major histocompatibility between donor and recipient as well as the degree of acute ischemic damage caused by hypoxia and reoxygenation of the kidney [Zager *et al* 1994]. Since major histocompatibility can be matched between donor and recipient by either serology or PCR commercial kit. Ischemia/reperfusion poses a major cause of renal dysfunction and delayed graft function after transplantation. The damages may either from hypoxia or the generation of free radicals from oxygen-rich reperfusion. To minimize the adverse effect of ischemia/reperfusion, it can be improved by: (1) limiting ischemic hypoxia injury or (2) protecting kidney from offensive ingredient in the reperfusion [Bonventre & Weinberg 1992].

Despite vastly reports on ischemia/reperfusion in kidney, the mechanism which leads to renal deterioration and organ failure remain unclear. In our previous study, the therapeutic effect of free radical scavengers suggested reactive oxygen species play a significant role in renal function after ischemia/reperfusion [Chien *et al* 1999]. Reactive oxygen species (ROS) act as second messenger during myocardial adaptation to ischemia and tyrosine kinase and MAP kinases are the targets for (ROS) generated in the preconditioned myocardium [Das *et al* 1999]. Because p38 MAP kinase might be upstream of NF kappa B, its activation appears to be important in the translocation and activation of the nuclear transcription factor NFkB. In turn, a variety of stress-inducible genes may be induced by NFkB.

ROS are well-known to trigger apoptosis in various cell types [Jacobson 1996]. Using an experimental model in our laboratory, we found that apoptosis of renal tubular cells could be induced in rats by no less than 45-minute ischemia followed by 1-4 hour reperfusion. Tubular apoptosis can be detected by several advanced techniques including DNA ladder, TUNEL, gene expression and Annexin V detection [Chien *et al* 1999]. Accumulative reports

have suggested the importance of c-Jun N-terminal kinase (JNK) and its substrate c-Jun in the signaling pathways to apoptosis. For example, the exposure of cells to apoptotic stimuli, including ultraviolet light, g-irradiation, tumor necrosis factor-a, and ceramide triggers JNK activity. When negatively inactivation of either SEK1, JNK, or c-Jun can prevent certain apoptotic process [Chen *et al* 1996, Butterfield *et al* 1997, Sluss *et al* 1994, Verheij *et al* 1996].

Heat shock protein have been shown to interact with other transcription factor in several signal transduction systems. The steroid and dioxin receptors are bound to hsp90 through their hormone-binding domains, and several of them must bound to hsp90 to have a ligand binding site exposed. The binding of ligands to these transcription receptors promotes their dissociation from hsp90 which exercise their fist step in corresponding signaling pathways. Several protein kinases, including the Src and Raf components of the MAP kinase system, are also bound to hsp90 [Pratt 1997]. Apoptosis also have strong correlation to hsp90, recent report from our laboratory showed retinoic acid-induced apoptosis reduced hsp90 expression after 6 hours of retinoic acid treatment [Wang *et al* 2000]. A RAR-a specific antagonist RO41-5352 successfully compromised the effect of retinoic acid inhibition in a lymphoma cell line.

In this project, we will try to clone all tyrosine kinase genes in rat kidney by specific degenerated protein tyrosine kinase (PTK) PCR primers. The putative PTK conserved region will be cut out and cloned into plasmid vector. The vector will be used to sequence all PTK gene fragments. GCG database will be used for sequences comparison. It is estimated that about one third of the PCR product are actually serine/threonine kinases. When all the cloned were identified and dot blotted onto nylon membrane, the ischemia/reperfusion kidney mRNAs will be used to reverse transcribed complementary probes and hybridized to the PTK membrane. In each treatment, we expect to have different pattern of PTKs.

PTKs and phosphatases play key roles in various cellular responses including mitogenesis, adhesion, differentiation, transformation, and apoptosis. Previous literature suggests that tyrosine phosphorylation/dephosphorylation-dependent signal transduction can be modulated by the intracellular redox status [Monteiro and Stern 1996]. In general, tyrosine kinase activity is stimulated by ROS directly or indirectly. For example, H_2O_2 stimulates phosphorylation of tyrosine residues of the insulin receptor. In contrast, tyrosine phosphatase activity may be inhibited by ROS. Administration of sodium nitroprusside (nitric oxide releasing agent) to HEK293 cells induced JNK1 and JNKK1 (SEK1) activity. However, JNK1 activity can be compromised by N-acetyl cysteine or NG-nitro-L-arginine (nitric oxide synthase inhibitor) [Kim *et al* 1997]. Base on this observation, nitric oxide must activate JNKK1 (SEK1) before JNK. When cell encountered certain stress conditions Stress-Activated Protein Kinases (SAPKs), Extracellular Signal-regulated Kinases (ERKs), and JNKs will all be activated. Amino-terminal transactivation domain of c-Jun was used as substrate for SAPKs activity. In ischemia/reperfusion process, SAPKs was not activated

when ischemia alone, but after the subsequent reperfusion. The magnitude of SAPKs was ranging from the start of reperfusion to 5 minutes (4.6 fold), 20 minutes (7.6 fold), and 90 minutes (4.9 fold). In parallel, the ERK1 and ERK2 only increased about 1.3 fold 5 minutes after the reperfusion [Pombo *et al* 1994]. When using Madin-Darby canine kidney epithelial cells as model for ATP depletion and repletion treatment, the SAPKs profile and the time course was the same as ischemia/reperfusion. The SAPKs activities in both kidney ischemia/reperfusion and canine cells in depletion/repletion were quickly activated may trigger part of gene regulations in kidney ischemia/reperfusion, possibly enhanced the c-Jun transacting activity [Pombo *et al* 1994].

After the rat kidney PTKs cloning complete, we will challenge the PTKs panel dot blot membrane with complementary mRNA probes from every treatments (time course after ischemia/reperfusion, specific inhibitor of PTKs pretreatment, anti-oxidant, and free radical scavengers). According to the sequential changes of different probes, we will have a profile of how the kidney signal transduction pathway response to ischemia/reperfusion. By using the PTKs panel membrane, we will explore the possible PTKs blocker or inhibitor to modulate PTKs pathway. The final goal of this research is to find possible therapeutic intervention to preserve kidney function during transplantation or physiological ischemia/reperfusion damage and diseases. The PTKs panel membrane can be shared or exchanged to other research groups using rat kidney model.

Results and Discussions

According to the PTK conserved domain sequence, we have compared NCBI ' s Blastn database for 43 protein tyrosine kinase-related sequences from some 4000 colonies. All the DNA sequences are analyzed by DNAstar for their phylogenic relation. The sequences and the phylogenic map are list below.

Among the 43 sequences, two of them are complete homolog to human (include cAbl). The rest of 41 clones are under investigation.

Although 43 tyrosine or serine/threonine protein kinase are cloned in this project, there is preferentially expression in kidney among all published 100 protein tyrosine kinases. Subsequently using other organ for the tyrosine kinase screening should be able to complete the rodent homologous of human protein tyrosine kinases and the preferentially expression in each organs. The profile of rat PTK should help other researchers' using rat model.

Self-evaluation

The direct results expected in this project:

Prior to this project, we have detected the apoptosis during the time frame of renal

ischemia/reperfusion in the rat. We have found the best time to induce most significant apoptosis. A paired of degenerated PTK PCR primer will be used for human protein tyrosine kinase cloning and adapted to this rat kidney model. We have identified 44 different clones by using this primer (Figure 2). In the project, we will continue to explore and characterize PTK profile of ischemia/reperfusion kidney in every time frame. We will expect to have differential pattern of PTKs from control, ischemia/reperfusion, anti-oxidant pretreatment, and free radical scavenger pretreatment. From these interaction of signal transduction pathway, we hope to elucidate possible therapeutic interventions to minimize the damage of kidney during ischemia/reperfusion.

五、參考文獻

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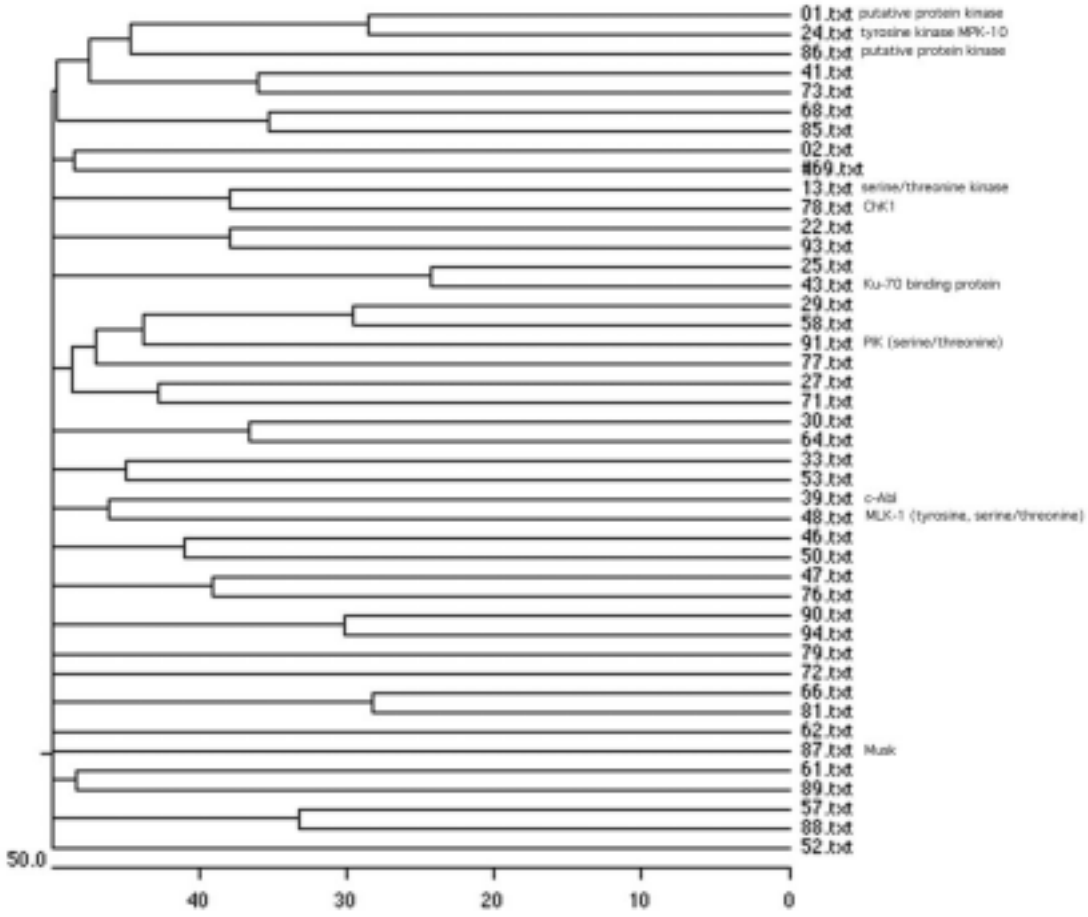
六、圖表



41.txt

GAATGCCCATACGTCATAGCCTTATNGTTGCCCATGTTCTTAAGTCTAATTAGAAAGCCAGTGG

TTAATGCCAAGATATGTATGCCATTACTGTACCTGTAGGGATATCATGCCTAT

CAGGCCATTGATATCAGGTCATTGATGTGGTACATAGACTATATATAT

43.txt

TGCCTTGCAAA....TAAATATTGAATTAATGAGCAGATGAATAGAGGATGGATGGATGGATGGAT

GGATGGATGG....ATGGGTAAATTGATGGGTAGATGGATACATGGGTAGATGAATGGATAG..ATGG

ATGGATAGATGGATGGATAAA....TAGGTG

46.txt

CCCAGC....TTTCTATGTTTGAGGACTGATAGGAAGGTAGCTCTGTGGCCTGNAGGGCACCTAGT

GC...CTGCATCTGAGAG.CCTTGTGGTAAGTAGTGCAG..AGGAATGGGAACATGAAGACCACTT

CGAGGA..TGGCA.....AGCACACATG.GG

47.txt

TACCAA....AGACTGGTGCTAGCCAACATTCAATGAACCATATGTTTACATAGACACTTCTTGAG

AAAT.TAACATGCGTATG.TGCAGTGGTGAGCAGATGACCTAATGGAAAGGCAATGGGTACACA
ATACAAAAGGGAATGCAAAGAGGTAGCTTCATTCT

48.txt .GC.TGGCACG....GGAA..TGGCAC.....CGGACCACCAAGA.....TGAGTGC...G....GC
GGGGACAT..ACGCTTGGATGG.CGCCTGAAGTCATCCGCGCTTCC..ATGTTT.....TCCAAGGGCA
.....GC.

50.txt .....GG.....AGGGAGGCAGAGACAAGA...GAAGGTCACAGGGCAG...CTG..NCCC
AGAG.CAC.ACAGTAGGTGGCAAAC..AAGGGAGTGG.GG
TATGCCTTTAACAAGGAGG..AAGTTGAGGA....GT.....

52.txt .....AAACTTAAATATGGTGCGGCTTCTATGGAAATNAGTT.....AGTNCCTCAAAAAG....
TTANCAACANAAGC.CGGTCATGGTGGTGCATGCCTTTAA.....TCCCAGCAC.....TCGGA
AGGTAGAANCAGGCA

54.txt
CGT.....AAGACTTCTGCTCCCCGT.C.CCGGACACTCTGGGTAGAAAAAT...ATGAAATACA.....TAT
ACTCCCGGAACTTCTGTA....TTCAGGA.....

57.txt
TTCTC...ATTCACAATGAACATGCAGCGCTCGGTGGTTTCTCCGGGTAAATGACACGTAA.GTGT.
TGTATTCAGGAACAGGATCTTATCA....C.CAGGGTGCACAGAGCAACCACG.....
..... AGCTC.GCAGATAGC.....C.TAT.

58.txt
AAAGAC.....AGTCT.....CCATG.....GTGTG.....GGATTCCTNGCC
TACTAA.....ATTG.TAACAGA.....GACGT.....TG

61.txt .....GCTTCA.....GGATAGCCCCC.....ATTCCAGTT...GTGNGGGACACACA..TAAACACTG
AGT.TGCACATCTGCTACATATGTGTGGGGAGGCCTAG.....GTGGGTCCTGAACAAC...TGAA
AC....AAGGCCTGTC.

62.txt
ACTCCTGGATGT.....GGTACTCTGT..CTTAAAAATGGTTGGGNGAGTGCTGTAGCTATGGCCAG...
AGAGACTCTTG....A...CAGTTGACCTGAGTAGCCTGAGATAAGACACCAGCAGCTATGAACAGA
CCCAGCTTCAGAACTTA.....CGAA

64.txt .....AT.CTTCAT..CAGCA.GAG.....AGAGG.....AA..GGT.....GCAG..AAACCCCAGNT.....
CCTTTGT.....CTTAGATTTT..CAGC..G..ATTAGACTG.TGTGTGCGGAGAA..AAGAGGATATA

66.txt
CGCAGA....GCCTAGG.GACAAGGGGGGATCAGGGGGAAAGGAGT.....GAGAC...CTTGAGGGA...
.....GG.....CTTTCGGA.....CAGGCCAG.GGTGGTCAG.....

68.txt .....TCAAACAAATCGGTCT....GCT.....AT...CT.....CT.....CAGTTCCCCT
TCAT.CCA...AGCTCTCAG..GGTAGAGGCAGAAGGAAGCCATT.....ATC.TTGC

71.txt .....AA....AAAACCTGACCCTATTAAGCA..CTACTTG..GCTTTCCAAGCTTTTCCCTAAA.
ATCTGG....GTG..CAAGCC.....TCCATG.ACCCCTGTGT..CTCTTCCATTTT..ATATGTCTGCAAACCT
AGCAACATA....TGGAT.

72.txt

A.....ACTTTGAGATG....GGACATGCAGGGTCTGTGTGCAC.TATATTCTGCACCTGTAGGGAGT
TTG...GGAA....CCTTCTCCAGGACCAAGTTAGACCAAGAAGTCTGAGGCCTCTCCCCATCTG.AC
TCTTGAGATGCTGTCGAGGG.T.AG
73.txt .....ATT.....GTAT.GCCACC.....ATT...ACT...G.GGGAGACAAGAA..CAAATG.....TCTA
CT.CA...GCCCAGAGAGG...GA.....ACTGGTGCAGA....C...CAAAGT....ACAGATAGTA
76.txt
T.....GGCTAGCGT.....AACATCTGATGGG.....GAA.....GGAGGGC.TTACAC.....TGGAGTT
GTGAGCTAG.GATATAGTG..ATGATAGAGGATA.....GAGGTTG....GGAATAG.....
77.txt
ACCA....ACAACTGAGGACGACGAAATGTTCAAATTCCCACACCTATAT.....GGNACATTCCT
CCCTTCAAA.....CCGTGGCATGGGATGTTCCCAGTGAGGTGAGAGATGCCCTTCACTGATGC....
.....TCTG
78.txt .....TTTGGCA.....ACAGTGTTT.....CG.GCATAATAATCGT.....GAGCGCTTACTGAAC
A....AGATGTGTGGGAC...TTTACCTTATGTCGCTCCAGAGCTTCTAAAGAGGA.AAGAATTTCAC
GCAGAACCAGT.T...
79.txt
TACTG...AAGACGTCTAGTCCCCTTGT.TCTAGCAGTGGGAGCATGAGAACTCAAAGAGATCCA
CCTGGCTTTGCCTCCCAAGCCTTGGGA....TTCAGAGTGTGCACCACCATACATAGCTCCACTTT
TATT.....TATATG
81.txt
CAAAGA....AGCTAAGTAACAAGGAGGGCCCAAGGGAGGATGTGT.....GAATCTCACTCAGAAG
A.....GGAAA.TAAAATAGCCATTGGAGG...TAGGAGAAAGGTGGGGAG.....ACAAAGGAAAT..
..AGGGAGT.....CA....
85.txt
C.....CA....CAACCCTGACATC.TCAAG.GATCTGCC.....CTCATTTGGCTNTCTT.....TANCTGT....NG
AGGAAGAT.....TCCACAACCATNTNTGGT.ATCCTTTATTC.....TAAAGCCAGAATCA.....TGG
G.G
86.txt ...AGA....AC.....TCTTGCCA....TCATATGGCT..GCTGTAATACC.....AG...TT.....CAGGGG
CA...TTAAAGCCCCTGGTTCCAC..AGTGCCTACTCAG.TAATGTACCAGGTCTGCATT.....TGACT
GC.CAGG
87.txt
TG.....ACTCCGTGGTGTAGCGGTTGTAGAAGAT...AGACTCGGGTGGCAT.CCAGCGG.....ATA
GGTATAGCATCGTT...TCCATCAG....CTTTG.....TAG
TAGTCTGCGGA..GTAGAT.....GTTCCTAG...AGAGG
88.txt
GG..A...ATTCTATATACACTTGCAGC.....TGA.....GACATGTGA....T.TAAA..AAGCAACAGTA
ACTCTTC.....C.CAGGGTCTACCT..CAGTTTGAAGAAT.ATGTCTGTC.....T.CA..
89.txt
GAGTAAATAT.TTTGGTTTCCTCA.AATTACCCAGTTGTTC...TTCAA..GATA...CAGCAG..AAGGAT

TACAT.....TTCTTGTAT..GTGGTGA...CTTGGATGTGAACAGCCCA..GGTAGATTCCTACACTTG
AATTTTTAGTAGGATTGT

90.txt

TCCA.....TGGGAAGG..CACTA.ATGTTC.....TCCTAGTTTTCCTCAGCG....CTTATCACTGGA
A..A...ATACAGAG.GGAGACCCAAGAA.....TGCCCGTGAGCA.GTGCACAGAGT..ACACACAA
GTTT.....

91.txt .....C.....ACC.....TCAA..AG..CTGTG.....TCCCTTCTTGCTCAGCAC...CT.....CGGGAGC
T...ATGTAGTTAGGAGTGCCAC..ACAGGGTCTTCTTC...CGTTCCCCTTCAT.ATTCC..ACTTTTGTT
GC.CAGG

93.txt .ATGC...ATACAC.ATGCACACA.TGCTTTCAGACAGCTGCTCCCCAGAGTAAGA.....ATGT
TTAGCTCTG.....GGACTTTAT.CT....TTCTTGGAGCAAATGGCAGATGTAAGCTT..TTGGCA.C.....
.....TATG.G

94.txt

CTCACACACTAC.....ACTGCGTAGT..CCCTACAGGCCCGTGTGGGTTCTTATCTCCCCAAGCTT...
CTTAGCACCTGAGTTA...TCACAGAGTAGAATCCCAAGAAAGGACAAGTATACTTGTGTGTACA
CACTCAGGATTGGTTCAGGAGACT.ACAA

#22-new.seq

TGTACCTGTTACTCTGTAGTTACTGCAGTCTTACACATCTGTACCTGTTACTCTGTGGTTACTGC
AGTCTCACACATCTGTACCTGTTACTCTGTGGTTACTGCAGTCTCACACATCTGTACCTGTTAC
TCTGTGGTTA.....

#24-new.seq

GATTGCCTGGCCATCGTATCCTTTAAAATCAAATAGTTCTGGAGCACAGTAAGGGAGGGTCCC
ACATAATGTTATTAACTTCTGACCCATGACTATCTTGGTGCTCAAT.....
.....

#25-new.seq

AAGTAACAGAGCACCAGAACAGGAGTTGACAGCTGGAGATCAAAATCAGCCATGGAGCTAAT
GAAACCTGGACCAGTGGATGCGTTTTG.....

#27-new.seq

AAAGAAAAAATTCTTACAGTGTCAGGAGTCACGGAGAAACCCAAGTCCAGCCCTTGGATTTC
AGTGGATTAGTCTCGGATAACCTCAGCACA.....

#29-new.seq

CAGGGTCTTCCGTTTCTGTTTCTCAGCAGTAACACAGCCCTGATGGCCCGTTTGTTCTGTGAG
GTTTTCAGACCATCAGGATCACAGAGCACTGACCATTCTTCACAGCATCCAAGGTTTTTAAAA
CCAAAGCATGCAATATGACAACACGACC.....

#30-new.seq

GGAGCAGAGCGCCTGGCTTCTTCGTCCAGCATTTTGAGCCCCGTCACTGCTGCTGTGTGCGAG
GGCTCCTTTTCATTGTGCAAGAGCTCCCTGCCGTATGGATAGACCATAATTTGTTTATCCATCTG
TCTGTTGAAG.....

#33-new.seq

TAAACCTACTGGGGCCTCTTAAGGATTAGGTGCGTCTTCTCTGATTGAACACAGACCAAAGAC
TACTCTGCTGTATATGTGTTGGGGGCCTTATATCAGCTGTTGTATGATAACTGGTGGGTGGG.....
.....
#39-new.seq
CCTAAGCAGGTTGATGACAGGGGACACCTACACAGCCCATGCTGGAGCCAAGTTCCCCATCA
AGTGGACTGCACCCGANAGCCTGGCCTATAACAAGTTCTCCATCAAGTCG.....
.....
#01-new.txt
AAATGGGCGGCCCTCATATTCCTCAGCTTCAAAGAATTCTGGCGCACAGTATGGCAGCGTGCC
ACAGAAATCAATCAGCTTCTGCCCAGGAAGTGTTTTAGCAGCTAGG.....
.....
#02-new.txt
GAATGGCAAAGTGGTAGCACTAAATTTTGCATCTTTGAGAGCTTGTAATTTTCAGAGTGCGTG
CGTGTGTGGCCAGATCCAGGTGAGCAATGCTGTGGTTGGATGTG.....
.....
#13-new.txt
CTTTAGTAACGAATTCACCTTTGGGAACAAGCTGGATACTTTCTGTGGTAGTCCTCCTTATGCT
GCCCCAGAACTCTTCCAGGGCAAAAAGTATGACGGTCCTGAAGTG.....
.....