90 年度計畫執行成果報告

不同蕈毒類藥劑對抑制近視眼球生長之影響(3/3)

NSC 90-2314-B002-184

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Introduction

The prevention and treatment of myopia are important issue of public health in many countries, especially in Taiwan where the prevalence rate of myopia is extremely high. The most important complication of extreme myopia is retinal degeneration affecting the posterior pole that is associated with elongation of ocular axial length. Unfortunately, the actual mechanism of the development of myopia is still unknown

Various pharmacological agents have been tried to treat myopia. Anti-cholinergic agnets, such as atropine and pirenzepine were reported to be effective to prevent the progression of myopia. However the actual mechanism of these agent is still unknown. We hypothesized that atropine and pirenzepine could prevent the progression of myopia through influencing the expression of growth factors in retina-RPE-choroidal complex and sclera. In this project, the effect of 1% atropine and 2% pirenzepine on the expression of growth factor mRNA from the retina-RPE-choroidal complex and sclera will be examined in the chick model of form deprivation myopia.

Subtraction-hybridization PCR method is used to selectively amplify target cDNA fragments and simultaneously suppress nontarget DNA amplification (45, 46). It can achieve greater than 1000-fold enrichment of differentially expressed cDNAs (ie. cDNA from myopic and control eyes). The basic idea of subtraction-hybridization PCR is those tracers DNA (in our experiment, the cDNA from myopic eyes) will primarily reassouciate with excess driver DNA (in our experiment, the cDNA from control eyes) while target sequences having no counterparts in driver will inevitably reassociate with each other, or remain single-stranded. The reassociated fragments common for driver and tracer are discarded, and the remaining DNA enriched in target sequences is cloned and analyzed. With this method, Ishibashi et al. showed the upreglation of crystalline mRNAs in form-deprived chick eyes (47). Den Hollander and coworker used this technique to isolate and map the novel candidate genes for retinal disorders (48). It is proven that subtraction-hybridization PCR is a powerful tool to study chick myopia.

chloroform, shaking for 15 minutes, then cooling at 4°C for 5 minutes. The suspension was centrifuged for 15 minutes at 4°C and the aqueous phase transferred to a new tube. The RNA was precipitated by adding 600 µl isopropanol, incubating on ice for 15 minutes, and centrifuging for 15 minutes at 4°C. The RNA pellets were washed once with 1 ml 75 % ethanol, dried, resuspended in 20 µl diethyl pyrocarbonate (DEPC)-treated water and incubated for 10 minutes at 60°C. The RNA was stored frozen at -80°C. RNA purity was determined by measuring the OD_{260}/OD_{280} and RNA quantity was estimated from OD_{260}.

5. Reverse transcription

One microgram of total RNA in 10 µl of DEPC-treated water was added to 10 µl of reverse transcription mixture consisting of 2 µl of 10X polymerase chain reaction (PCR) buffer (500mM KCl, 100mM Tris-HCl pH9, 15mM $MgCl_2$, 0.01%(w/v) gelatin), 4 µl of 5mM dNTP, 1 µl of Maloney Murine Leukemia Virus reverse transcriptase, 1 µl of random hexamers, 0.5 µl of RNasin, and 1.5 µl of DEPC-treated water. This mixture was incubated for 45 minutes at 37°C, and then 5 minutes at 90°C. cDNA was stored frozen at -80°C.

6. Subtraction-hybridization PCR method

6.1. Driver Preparation

Double-stranded cDNA will be synthesized from 1µg of poly(A)+ RNA of the control eyes using the Great Length cDNA synthesis kit . To prepare cDNA fragments suitable for efficient hybridization, 100 ng of double-strand cDNA will be digested with RsaI, phenol extracted, ethanol precipitated, and then ligated with an Rsa-adapter (which had one blunt end) in 10 µl mixture containing 2µM Rsa-adapter and 1X ligase buffer. Ligation will be carried out by adding 1µl of T4 DNA ligase (1u/µl) and incubating for 16 h at 16 ℃ . In 500µl of PCR, we will use 1.0µl of ligation mixture, 50 µl of PCR 10X buffer, 20 µl pf Rsa-primer (10 µM), 20 µl of 25 X Mix of thermo-stable polymerases, and 10 µl mixture of dNTPs (10 mM each). The 25 X mix of thermostable polymerases will be prepared using 100 µl of Klen Taq DNA polymerases (25µ /µl), 6 µl Pfu DNA

polymerase(2.5μ /μl), and 300 μl of TaqStart antibody. The PCR mixture will be heated for 7 min at 75 ℃ and PCR will be performed as follows: denaturation, 95 ℃ ,5 s; annealing, 68℃, 30 s; synthesis, 72 ℃ ,1.5min. After 15 cycles of PCR, the amplified cDNA from the reaction mixture will be phenol/choloform extracted and ethanol precipitated. The pellet will be dissolved in 10 μl of TE buffer. The Rsa-adapter will be removed from amplified cDNA with RsaI and the digest will be purified using Chroma Spin-100 Columns.

6.2. Tester Preparation

One hundred nanograms of myopic eyes-amplified cDNA(prepare as a driver) will be ligated with Adapter 1 or Adapter 2 in separate 10-μl ligation reactions containing 2 μM Adapter 2. Samples will be then heated at 70 °C for 5 min to inactivate the ligase and stored at –20 °C.

6.3. Subtractive Hybridization

For the first round of the subtractive hybridization, we will mix in different tubes 3 μg of driver cDNA with 100 ng of tester Adapter 1-ligated tester cDNA or with 100 ng of tester Adapter 2-ligated tester cDNA and ethanol precipitated at the mixture. Pellets will be resuspended in 1.5 μl of hybridization buffer (50mM Hepes, pH 7.5, 0.5M NaCl, 0.02mM EDTA). The solutions will be overlaid with mineral oil, DNA will be denatured (1.5 mM, 98℃), and samples will be allowed to anneal (20 h, 68 ℃). Then 1.5 μl (1.5μg) of fresh denatured driver cDNA in hybridization buffer will be added in both samples. After 20 h of additional incubation at 68 ℃ two hybridizing samples will be mix together and incubated for 20 h at the same temperature. The hybridized DNA will be diluted in 200 μl of diluted buffer (50mm Hepes, pH 7.5, 50 mM NaCl, 0.02mM EDTA) and heated at 75 ℃ for 10 min.

6.4. Selective PCR Amplification

In 25 μl primary PCR, we will use 1.0μl of diluted subtracted cDNA, 2.5 μl of PCR 10X buffer, 1.0 μl of PCR Primer 1(5 μM), 1.0 μl of 10X PCA Primer 2(5μM), 1.0 μl of 25X mix of thermostable polymerases, and 0.5 μl of dNTP (10mM). The mixture will be heated for 7 min at 75 ℃ and 30 cycles of PCR (95 ℃ , 5 s; 68 ℃ ,30 s; 72 ℃ ,1.5min) will be conducted. One microliter of primary PCR mixture will be diluted 10-fold and used in 10% secondary PCR

cycles, in which we will replace PCR primers 1 and 2 with Nested PCR Primers 1 and 2.

6.5. Cloning and Sequencing of Difference Products

The final difference product will be digested with RsaI and ligated into pTZ18R. Double-stranded plasmid will be prepared using Wizard Minipreps columns and sequenced with *fmol* DNA sequencing system. Resulting sequences will be compared to the Gene-Bank database using the FASTA program.

6.6. Southern, Northern, and Dot Blot Analysis

For Southern blot analysis, amplified tester, dreiver, and subtracted cDNA (300ng per track) will be run on a 1.5 % agarose gel and transferred to Hybond-N filters. The filters will be hybridized with 32 P-labeled IL2R and G3PDH cDNAs that will be made using human IL2R and human G3PDH control amplifier sets. For dot blot analysis, amplified tester and driver cDNA were spotted (200 ng per spot) on the Hybond-N filters and hybridized with 32P-labeled amplified inserts from subtracted clones and a β-actin probe amplified using the human β-actin control amplifier set. For Nothern blot analysis, poly(A)+ RNA from the myopic and the control eyes (3μg per lane) will be fractionated by 1 % glyoxal agarose denaturing gel electrophoresis and transferred to the hybond –N filter that will be used for hybridization with 32P-labeled amplified inserts of subtracted clones.

Results

To isolated genes up-regulated in the sclera of form deprivation chick eyes, a cDNA library was generated by a suppression subtractive hybridization methods based on the suppression PCR effect which is capable of isolating differentially expressed genes in one population (tester) but not in the other (driver). PolyA RNA was extracted from the sclera of myopic chick eyes (tester) and control eyes (driver) at day 15 after form deprivation treatment. Each polyA RNA was synthesized into tester and driver cDNA. Suppression subtractive hybridization was performed as described in the materials and methods section. Final PCR products were cloned into T/A vectors to creat the cDNA library. For cDNA library screening, probes synthesized from clones randomly picked from the library were subjected to Northern blot analysis. As a result, 6 out of 400 clones were isolated as a cDNA fragment of up-regulated genes in the form deprivation eyes samples. The insert of 6 clones was

sequenced and subjected to homology search with the Genebank, EMBL, and DDBJ DNA database. Siw genes were identified, including angiopoietin-2 gene, caspase 6 gene, prkdc gene for DNA-dependent proteinase kinase, retrotranspon, and alpha1 integrin gene. The significance of these genes in the process of development of myopia is under investigation

NCBI

results of BLAST

BLASTN 2.2.3 [Apr-24-2002]

H12

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1028791878-014970-6042

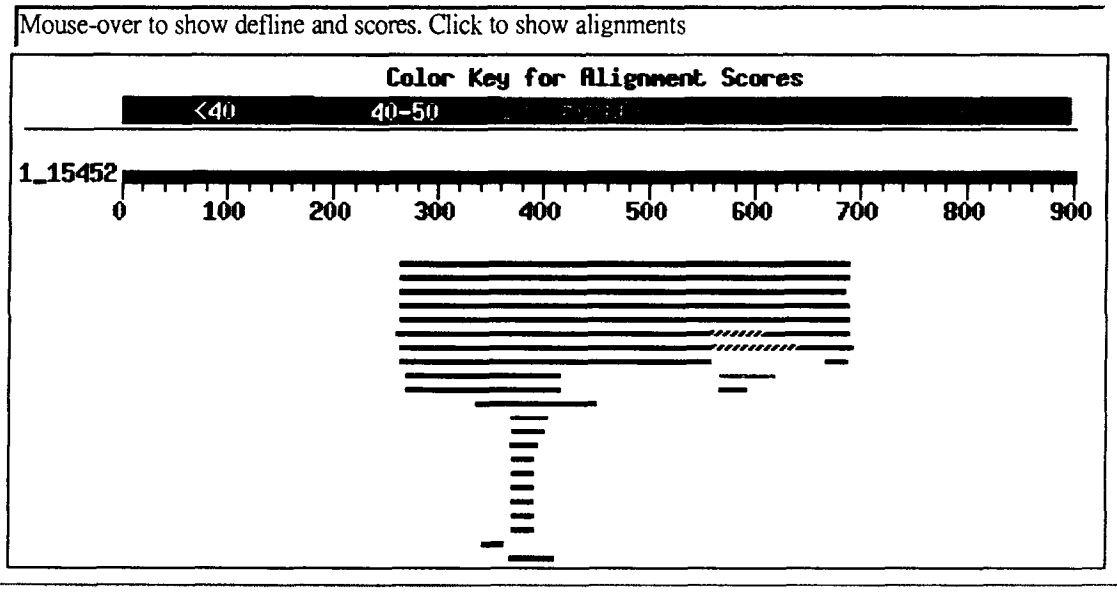
Query=
(901 letters)

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences)
1,377,342 sequences; 6,271,812,311 total letters

If you have any problems or questions with the results of this search please refer to the BLAST FAQs

Taxonomy reports

Distribution of 27 Blast Hits on the Query Sequence



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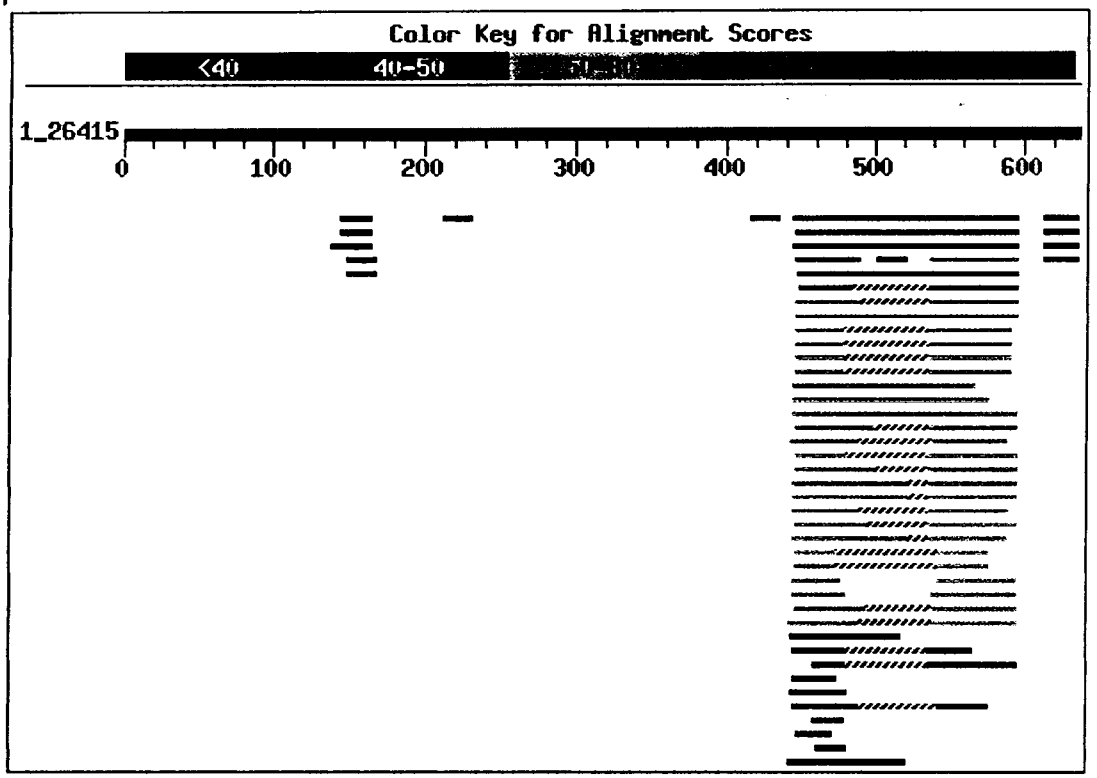
gi|1347215|gb|L22146.1|CHKPOLLIA	Gallus gallus POL-like gene...	165	1e-37
gi|1347220|gb|L22149.1|CHKPOLLID	Gallus gallus POL-like gene...	157	3e-35

Mouse-over to show defline and scores. Click to show alignments



RID=1031123539-01695-1701,

Sequences producing significant alignments:	Score (bits)	E Value
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gi|21465372|gb|AC092403.4| Gallus gallus clone WAG-100N11, ...	115	7e-23
gi|4028140|gb|AF082667.1|AF082667 Gallus gallus class II cy...	84	2e-13
gi|4995211|emb|AJ012570.1|GGA012570 Gallus gallus DNA seque...	84	2e-13
gi|14530846|gb|AC084761.2| Gallus gallus clone WAG-69H2, co...	82	1e-12
gi|12545295|gb|AC084760.2| Gallus gallus clone WAG-65N20, c...	80	4e-12
gi|2331057|gb|U88211.1|GGU88211 Gallus gallus retrotranspos...	80	4e-12
gi|341819|gb|M28069.1|CHKCR1A Gallus gallus vitellogenin ge...	80	4e-12
gi|3472226|gb|L22152.1|CHKPOLLIG Gallus gallus reverse trans...	80	4e-12
gi|3472218|gb|L22148.1|CHKPOLLIC Gallus gallus POL-like gene...	80	4e-12
gi|63883|emb|Y00324.1|GGVITCR1 Chicken vitellogenin gene 3'...	80	4e-12
gi|20066274|gb|AC091091.2| Gallus gallus clone WAG-93J15, c...	78	1e-11
gi|62880|emb|X56659.1|GDCOL6A2G Chicken Col6A2 gene for typ...	76	6e-11
gi|19526116|gb|AC091726.2| Gallus gallus clone WAG-126P17, ...	74	2e-10
gi|3927807|gb|L17432.1|CHKHBBRE Gallus gallus rho-globin, b...	74	2e-10
gi|16303289|dbj|AP003796.2| Gallus gallus genomic DNA, chro...	72	9e-10
gi|3472220|gb|L22149.1|CHKPOLLID Gallus gallus POL-like gene...	72	9e-10
gi|3472217|gb|L22147.1|CHKPOLLIB Gallus gallus POL-like gene...	72	9e-10
gi|15022025|gb|AC091725.2| Gallus gallus clone WAG-105M15, ...	62	9e-07
gi|15487991|gb|AF405540.1|AF405540 Gallus gallus lysozyme p...	62	9e-07
gi|15487987|gb|AF405538.1|AF405538 Synthetic construct lyso...	62	9e-07
gi|211540|gb|K02907.1|CHKCM14 Chicken CR1 repetitive elemen...	62	9e-07
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gi|13364843|dbj|AB042324.1| Gallus gallus CENP-C gene for c...	60	4e-06
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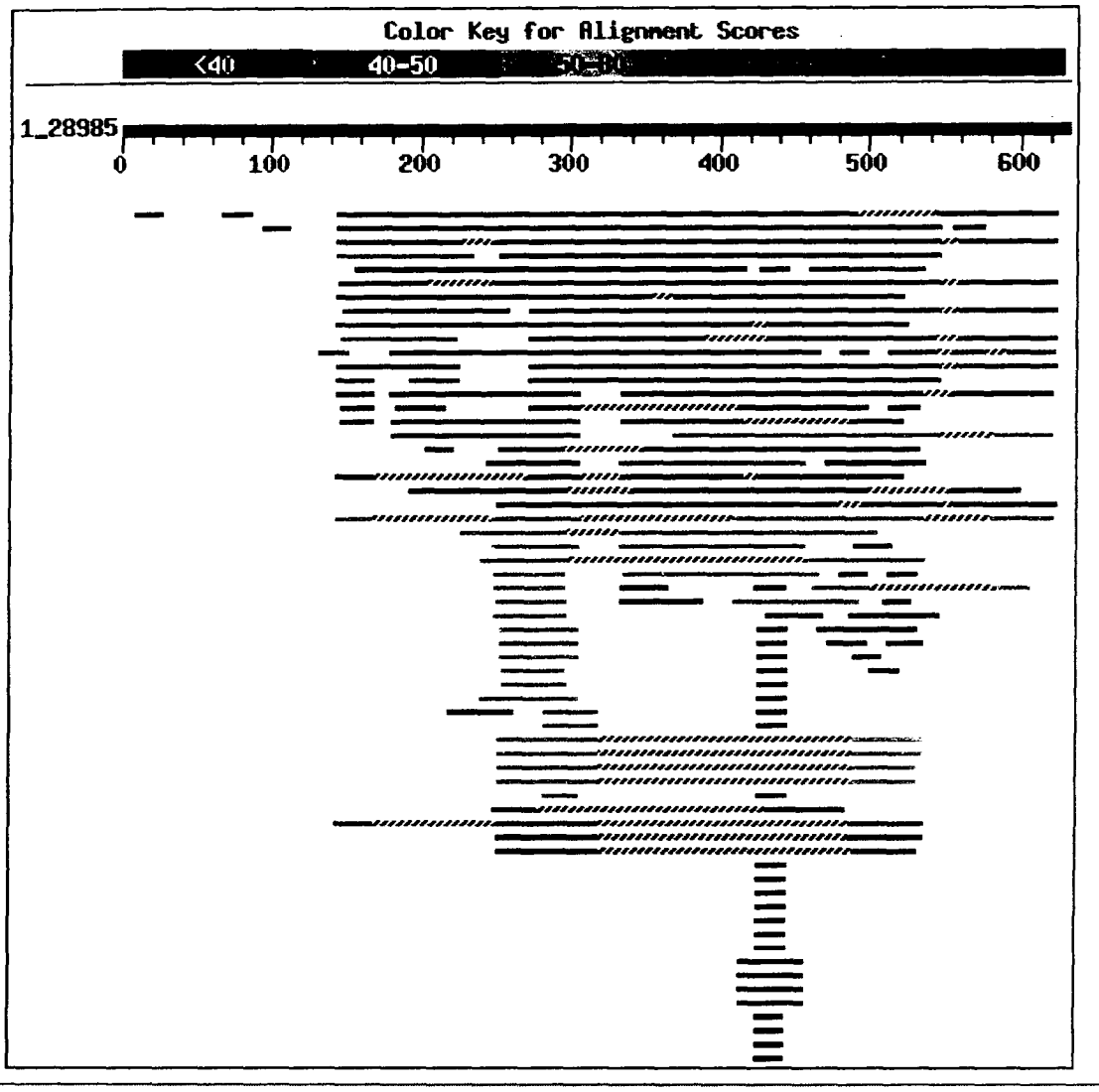

Sequences producing significant alignments:	Score (bits)	E Value
gi|18920651|gb|AF469049.1| Gallus gallus caspase-6 gene, co...	204	9e-50
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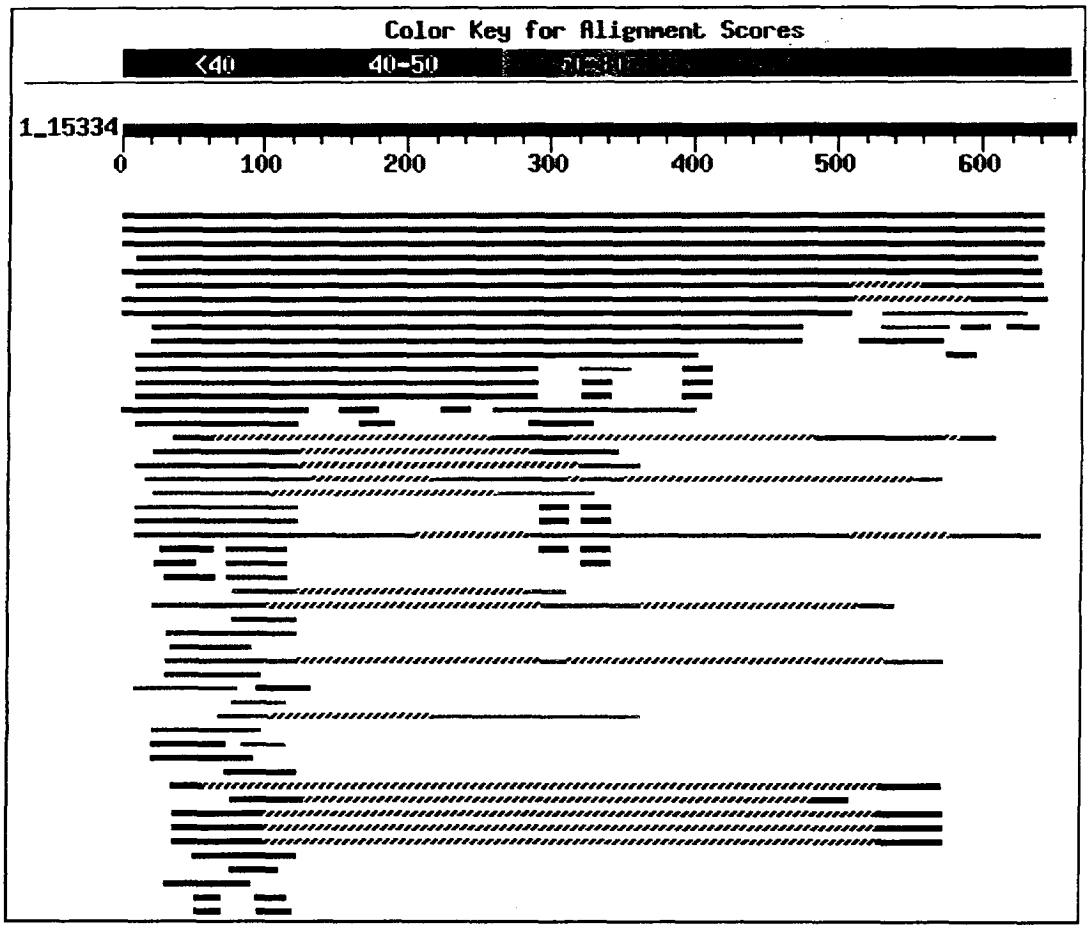
gi|21553214|gb|AC092081.3| Gallus gallus clone WAG-77D19, c...	168	5e-39
gi|18057089|gb|AC094011.2| Gallus gallus clone WAG-50C6, co...	167	2e-38
gi|4584404|emb|AJ012220.1|GGA012220 Gallus gallus ip3ka gen...	165	8e-38
gi|20334954|dbj|AB028136.1| Gallus gallus prkdc gene for DN...	155	8e-35
gi|14530846|gb|AC084761.2| Gallus gallus clone WAG-69H2, co...	143	3e-31
gi|13195247|gb|AF308605.1|AF308605 Gallus gallus clone CC n...	143	3e-31
gi|21465372|gb|AC092403.4| Gallus gallus clone WAG-100N11, ...	139	5e-30
gi|13195244|gb|AF308540.1|AF308540 Gallus gallus clone H3 C...	139	5e-30
gi|15528861|gb|AC093704.1| Gallus gallus clone WAG-38H9, co...	137	2e-29
gi|13195248|gb|AF308606.1|AF308606 Gallus gallus clone CW n...	137	2e-29
gi|13195246|gb|AF308604.1|AF308604 Gallus gallus clone j4a ...	137	2e-29
gi|21686442|gb|AF523666.1| Gavia immer clone TUMWGi7.1.98 m...	131	1e-27
gi|14595212|gb|AF303092.1|AF303092 Tetrao tetrix tetrix clo...	105	6e-20
gi|63508|emb|X04961.1|GGHSP108 Hen gene for steroid inducib...	98	2e-17
gi|63425|emb|X61001.1|GGGTLYSO G.gallus gene for goose-type...	94	2e-16
gi|63002|emb|X61197.1|GG325GEN G.gallus mRNA (clone 325gen)	94	2e-16
gi|455116|gb|U06050.1|APU06050 Anas platyrhynchos deltal-cr...	92	1e-15
gi|211944|gb|M31321.1|CHKHSPA Chicken 108K heat shock prote...	86	6e-14
gi|20066274|gb|AC091091.2| Gallus gallus clone WAG-93J15, c...	82	9e-13



Sequences producing significant alignments:	Score (bits)	E Value
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gi|347226|gb|L22152.1|CHKPOLLIG Gallus gallus reverse trans...	656	0.0
gi|347215|gb|L22146.1|CHKPOLLIA Gallus gallus POL-like gene...	585	e-164
gi|347218|gb|L22148.1|CHKPOLLIC Gallus gallus POL-like gene...	573	e-160
gi|347220|gb|L22149.1|CHKPOLLID Gallus gallus POL-like gene...	559	e-156
gi|347217|gb|L22147.1|CHKPOLLIB Gallus gallus POL-like gene...	458	e-126
gi|12545295|gb|AC084760.2| Gallus gallus clone WAG-65N20, c...	444	e-122
gi|15022025|gb|AC091725.2| Gallus gallus clone WAG-105M15, ...	444	e-122
gi|341819|gb|M28069.1|CHKCR1A Gallus gallus vitellogenin ge...	365	4e-98
gi|63883|emb|Y00324.1|GGVITCR1 Chicken vitellogenin gene 3'...	365	4e-98
gi|20066274|gb|AC091091.2| Gallus gallus clone WAG-93J15, c...	268	8e-69
gi|15487991|gb|AF405540.1|AF405540 Gallus gallus lysozyme p...	180	1e-42
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gi|18419435|gb|AF410481.1| Gallus gallus egg white lysozyme...	133	3e-28
gi|4028140|gb|AF082667.1|AF082667 Gallus gallus class II cy...	103	3e-19
gi|211539|gb|K02906.1|CHKCM13 Chicken CR1 repetitive elemen...	96	6e-17
gi|15145070|gb|AC091708.2| Gallus gallus clone XXbac-68C5, ...	86	6e-14
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gi|14530846|gb|AC084761.2| Gallus gallus clone WAG-69H2, co...	80	4e-12
gi|13364843|dbj|AB042324.1| Gallus gallus CENP-C gene for c...	80	4e-12
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