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研究発表

- 学会誌等

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  H. Wada, Auditory mechanics, 第13回バイオエンジニアリング講演会，2001年1月16日

  K. H. Iwasa, Physical aspect of OHC motility, 第13回バイオエンジニアリング講演会，2001年1月16日

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Contents

1. Introduction

The ear is a highly sensitive and tuned mechanical system. In the ear, hair cells like other sensory receptors must detect stimuli at the lowest possible intensity. Unlike most other receptors, though, hair cells operate at their thresholds with far smaller energies – we probably hear sounds at intensities so low that they vanish into the internal ear's thermal noise. Psychophysical experiments confirm that humans can detect auditory stimuli that provide each receptor cell with energy near the thermal level (Hudspeth, 1997). Each hair cell thus has an energetic threshold only 1 % that of photoreceptor.

To address this problem, hearing in mammals depends on a feedback process within the inner ear termed the 'cochlear amplifier' (Davis, 1983). The mechanism involves a population of cells, the outer hair cells (OHCs) of organ of Corti, which transduce motion of the basilar membrane induced by sound and generate forces to cancel the viscous damping of the cochlear partition (Dallos, 1996; Mammano et al., 1993). OHCs alter the passive mechanics of the cochlea, enhancing both the sensitivity and the frequency selectivity of the auditory system. The molecular basis of the mechanism is thought to be a voltage-sensitive protein 'motor' embedded in the basolateral membrane of the OHCs (Kalinec et al., 1992). The unique motor are thought to be an OHC-specific protein and abundantly expressed in OHCs. However the protein has been unidentified yet, because of the limited numbers of the OHCs in a cochlea (3000 per cochlea).

To identify candidate genes for the unknown protein, we constructed a guinea pigs organ of Corti cDNA library and then randomly sequenced using recently developed techniques of the genetic engineering. Each of 197 EST was compared by FASTA 3.0 analysis with the known genes deposited in DNA databases to identify

ESTs derived from unknown genes. The tissue distribution of the unknown genes in different tissues was analyzed by RT-PCR to find cochlea specific genes. However, candidate gene of OHC protein motor could not be identified.

Recently, a novel gene "prestin" has been cloned from a gerbil cochlea cDNA library (Zheng et al., 2000). Prestin-transfected cells have revealed unique electrophysiological properties, suggesting that prestin is a potent candidate of the OHCs motor.

Therefore, after that, based on the base sequences of prestin deposited in the NCBI (National Center for Biotechnology Information; responsible for GenBank databese), an attempt was made to clone the gerbil prestin cDNA by polymerase chain reaction (PCR), which is a technique for amplifying the number of copies of a specific region of DNA *in vitro*.

2. Overview and background

2.1. Cochlear function and mechanism

The cochlea is a hydromechanical frequency analyzer located in the inner ear (Figure 2.1). Its principal role is to perform a real-time spectral decomposition of the acoustic signal in producing a spatial frequency map. The frequency analysis may be understood with the aid of Figure 2.2, which shows a straightened cochlea with a snapshot of its basilar membrane displaced in response to a pure tone. Upon delivery of an acoustic signal into the fluid filled cochlea, the basilar membrane undergoes an oscillatory motion at the frequency of the sound, resulting in a wave traveling toward its distal end. The drawing shows an instantaneous view of this traveling wave. The wave is spatially confined along the length of the basilar membrane, and the location of its maximum amplitude is related to the frequency of the sound. The higher the frequency, the more restricted the disturbance to the proximal end. The vibrating membrane supports the sense organ of hearing, the spiral organ of Corti (Figure 2.3), which is deformed maximally in the region of the peak of the traveling wave. In this location, the sensory receptor cells of the organ of Corti receive maximal mechanical stimulation, transduce it into maximal electrical signals, and thus produce maximal afferent sensory out flow from the cochlea. Thus, mechanical frequency analysis is performed by matching particular frequencies with particular groups of auditory receptor cells and their nerve fibers.

Understanding of frequency analysis in the inner ear progressed through three main epochs. The first was dominated by Helmholtz's suggestion that lightly damped, spatially ordered, mechanically resonant elements in the cochlea perform the spectral analysis (Wever, 1949). The second epoch, lasting from the late 1940s to the early 1970s, was dominated by von Bekesy's description of the travelling wave (von

Bekesy, 1960). The third epoch, during which a fundamentally different paradigm has emerged, was dominated by Dallos (Dallos, 1988). According to the paradigm, von Bekesy's traveling wave is amplified by the a local electromechanical amplification process in which one of the mammalian ear's sensory cell groups, outer hair cells (OHCs), function as both sensors and mechanical feedback elements. The ideas that the operation of a sense organ is dependent upon local mechanical feedback modification by what resembles a sensory receptor cell, that such mechanical feedback may operate at audio frequencies utilizing some novel cellular motor, and that the resulting modification of the receptor output is responsible for the remarkable performance of the systems, were certainly unexpected. The means of satisfying the demands upon the mammalian cochlea appear to be a non-linear local feedback process, which is assumed to result in a cycle-by-cycle boost of vibratory amplitude (Gold, 1948; Davis, 1983). The amplification preferentially functions at low signal levels, and confers high sensitivity and wide dynamic and frequency range on ear's operation.

2.2. Cochlear anatomy

Figure 2.1 shows a simplified sketch of the peripheral auditory system. Airborne sound entering via the ear canal propagates to the lateral boundary of the middle ear, the eardrum, which is set in vibratory motion. These vibrations are mechanically transmitted by the ossicles to the fluid-filled cochlea. In most mammals, the ossicles are a chain of three linked bones within the air-filled middle ear cavity. The inner ear comprised of a vestibular and cochlear portion is a system of cavities in the temporal bone of the skull. The entire cavity is fluid filled.

Key elements for understanding the neurobiology of the cochlea are situated

within the cochlear partition. This triangular shaped duct, shown in the cutaway of Figure 2.4, is the cochlear portion of the membranous inner ear. The latter contains the sensory epithelia of the auditory systems. The space between the bony and membranous portions of the inner ear is filled by fluid, the perilymph, while the membranous portion filled by endolymph. The boundaries of the cochlear partition (also known as scala media) are the basilar membrane (separating it from one of the perilymph-filled channels the scala tympani), Reissner's membrane (separating it from the other perilymphatic channel the scala vestibuli), and the lateral wall of the cochlea. The auditory sensory epithelium, the organ of Corti, is a cellular matrix located on the scala media side of the basilar membrane. As shown in Figure 2.5, a complement of support cells, inner and outer hair cells (IHCs, OHCs) form a unit segment, which is repeated about 3500 times along the length of the organ of Corti. OHCs rapidly change length on its membrane polarization. The contraction of the three rows of OHCs is thought to amplify mechanical inputs by accentuating the basilar membrane's motion (Figure 2.6). The hair bundles of OHCs, which are tightly connected to the tectorial membrane, may contribute to tuning and amplification. The bundles of the single row of IHCs are freestanding, and IHCs sense shearing movements of the endolymph beneath the tectorial membrane and transduce it into electrical signals, but probably contribute little to tuning.

2.3. Outer hair cells and protein motor

The outer hair cell (OHC), a unique embellishment of the mammalian cochlea, is essentially an elongated cylinder with a hair bundle at its apical end and a nucleus near its base (Figure 2.7). The OHC exhibits an unprecedented form of motility (Figure 2.8). Depolarization of the cell's plasma membrane causes the cylindrical

soma to shorten, and hyperpolarization leads to elongation (Brownell et al., 1985; Ashmore, 1987). These motions are associated with a specialization of the cell's lateral plasma membrane, which is endowed with several billion intrinsic molecule of an unknown protein (Gulley et al., 1977; Kalinec et al., 1992). Changes in membrane potential cause these molecules to contract or expand within the plane of the membrane, thus changing the hair cell's length. The mechanism is independent of ATP hydrolysis (Kachar et al., 1986; Holley and Ashmore, 1988) and calcium (Iwasa et al., 1995) and does not depend on microtubule or actin systems (Holley and Ashmore, 1988; Kalinec et al., 1992). The motor candidate is a voltage-sensitive molecule able to change area when the membrane potential changes, and can be observed as a high-density particle array in the basolateral membrane of the cell. Associated with the OHC electromotility is a charge movement detectable in whole cell (Ashmore et al., 1990; Santos-Sacchi, 1991), and in membrane patch recording (Gale and Ashmore, 1997) that is thought to arise from a dipole reorientation accompanying the conformational change of the molecule. And then, the unique motor are thought to be an OHC specific protein and abundantly expressed in OHCs.

The motor for this unique form of motility has been the focus of many recent investigations. Several candidates for the OHC motor molecule have been proposed. One possibility is that it is a modified anion exchanger linked to the cytoskeleton (Knipper et al., 1995; Kalinec et al., 1997). A second possibility is that the motor is a modified ion channel that retains its voltage sensor but has lost ionic permeability. A third possibility is that the motor molecule is an electroneutral transmembrane transporter, a sugar transporter GLUT5 (Geleoc et al., 1999). These candidates for the OHC protein motor have been proposed, however, the primary amino acid sequence of the protein motor had not yet been identified.

Recently, Zheng et al. indicated the gene that codes for a specialized protein

motor which produces the motility, and designated it "Prestin". Prestin-transfected cells have revealed unique electrophysiological properties.

2.4. Construction and analysis of a cDNA library

While a complete copy of the genome is present in every cell, only those genes that are important for the function of a particular cell are translated into proteins (Figure 2.9 and 2.10). The template for translation of a gene is messenger RNA (mRNA), an ephemeral nucleic acid with a half-life measured in hours, and the presence of mRNA provides a marker for the activity of genes. Increasingly, identification of genes sequences, and, thus, of encoded proteins, is made through the isolation and reverse transcription of mRNA species from a tissue of interest. This approach has been made possible by the development of methods for amplification of DNA sequences into quantities large enough for sequence analysis. Such amplification can be accomplished by cloning DNA fragments into bacterial replication vectors (Figure 2.11) or by the polymerase chain reaction (PCR) (Figure 2.12). The former method has the advantage that none of the sequence needs to be known. The latter method has the advantage to clone a known gene.

An important tool for the identification of genes expressed in a tissue is a cDNA library (Figure 2.13). Such a library is constructed by isolating mRNA from tissue and transcribing all of the mRNAs into complementary DNA (cDNA) copies using reverse transcriptase. The cDNAs are inserted into plasmids and cloned. Each clone obtained in this way is called a cDNA clones, and the entire collection of clones derived from one mRNA preparation constitutes a cDNA library. Replication of the plasmid vector in bacteria can then amplify the cDNAs to provide large quantities of DNA. Screening such a library by any of several different strategies can identify gene sequences expressed in the tissue from which the library was

constructed.

Recently, cDNA libraries have been constructed either from the cochlea (Robertson et al., 1994; Soto-Prior et al., 1997; Heller et al., 1998; Skvorak et al, 1999), from the organ of Corti (Wilcox and Fex, 1992), or from the OHCs (Harter et al., 1999). Sequencing of the randomly selected clones from a cDNA library and then analysis of these sequences termed expressed sequence tags (ESTs) has been shown to be an useful way of identifying of novel genes (Soto-Prior et al., 1997; Harter et al., 1999; Skvorak et al, 1999). ESTs provides short nucleotide sequences that act as unique identifier of both known and unknown genes by compared with the nucleotide sequences deposited in DDBJ (http://www.ddbj.nig.ac.jp/Welcome-j.html), which was designed to operate as one of the international DNA databases, including EBI (European Bioinformatics Institute; responsible for EMBL database) in Europe and NCBI (National Center for Biotechnology Information; responsible for GenBank database) in the USA as the two other members. And analysis of the tissue expression of the unknown genes using RT-PCR has proven a rapid access to cochlear-specific genes (Soto-Prior et al., 1997; Harter et al., 1999). This approach is thought to be efficient to identify candidate genes.

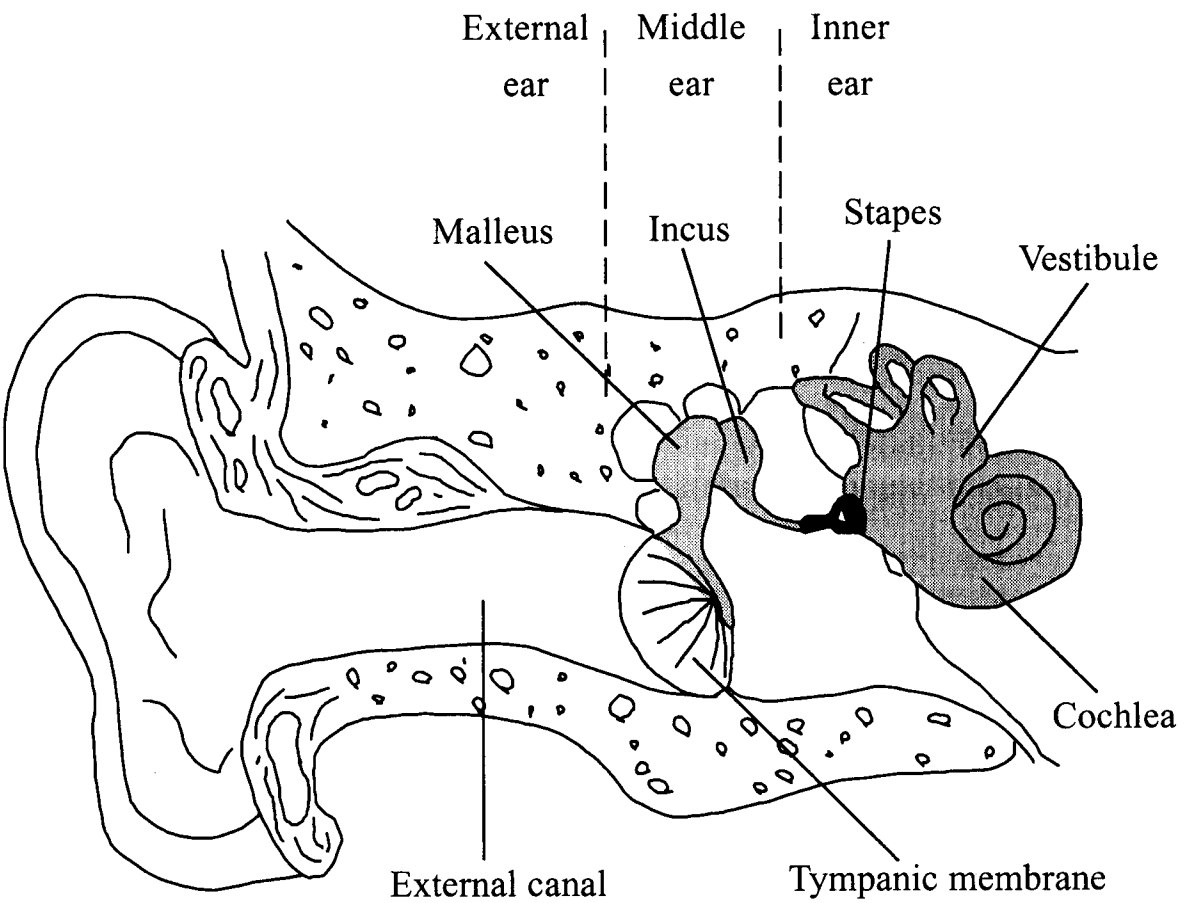


Figure 2.1. Outline of the auditory system of human. Sound is conducted to the tympanic membrane that sets the three ossicles of the middle ear into vibratory motion. The innermost ossicle, the stapes, delivers these vibrations to the fluid-filled cochlea.

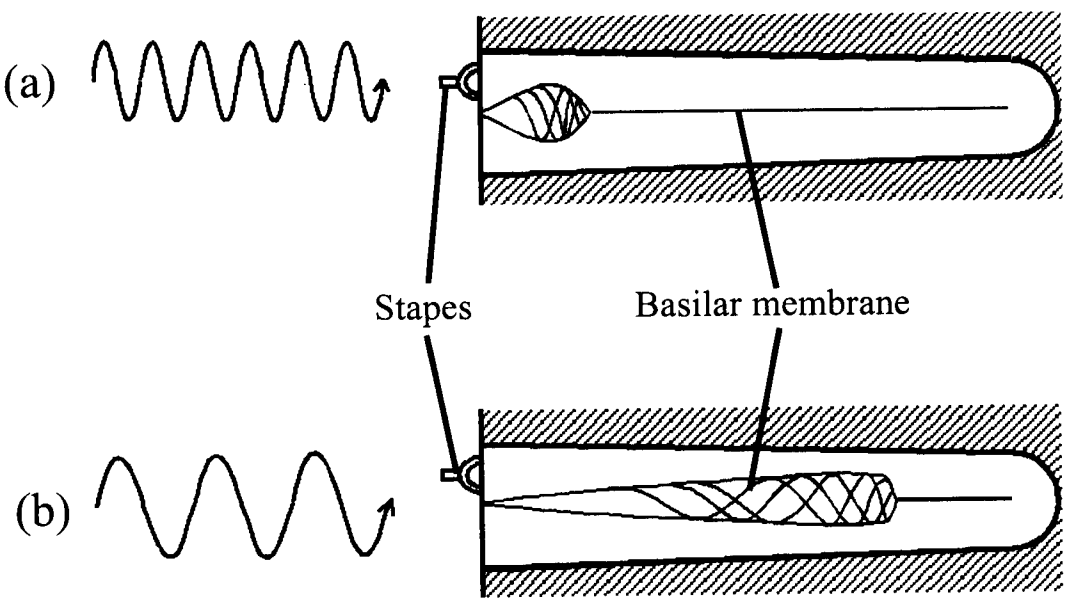


Figure 2.2. The vibration mode of the basilar membrane. The snail-shaped cochlea is straightened out, and one of its internal dividing boundaries, the basilar membrane, is shown as solid line. When a pure tone is transmitted to the cochlea, the basilar membrane undergoes an oscillatory motion at the frequency of the sound, resulting in a wave traveling toward its distal end. The location of its maximum amplitude is related to the frequency of the sound. (a) A high frequency sound is transmitted to the cochlea. (b) A low frequency sound is transmitted to the cochlea.

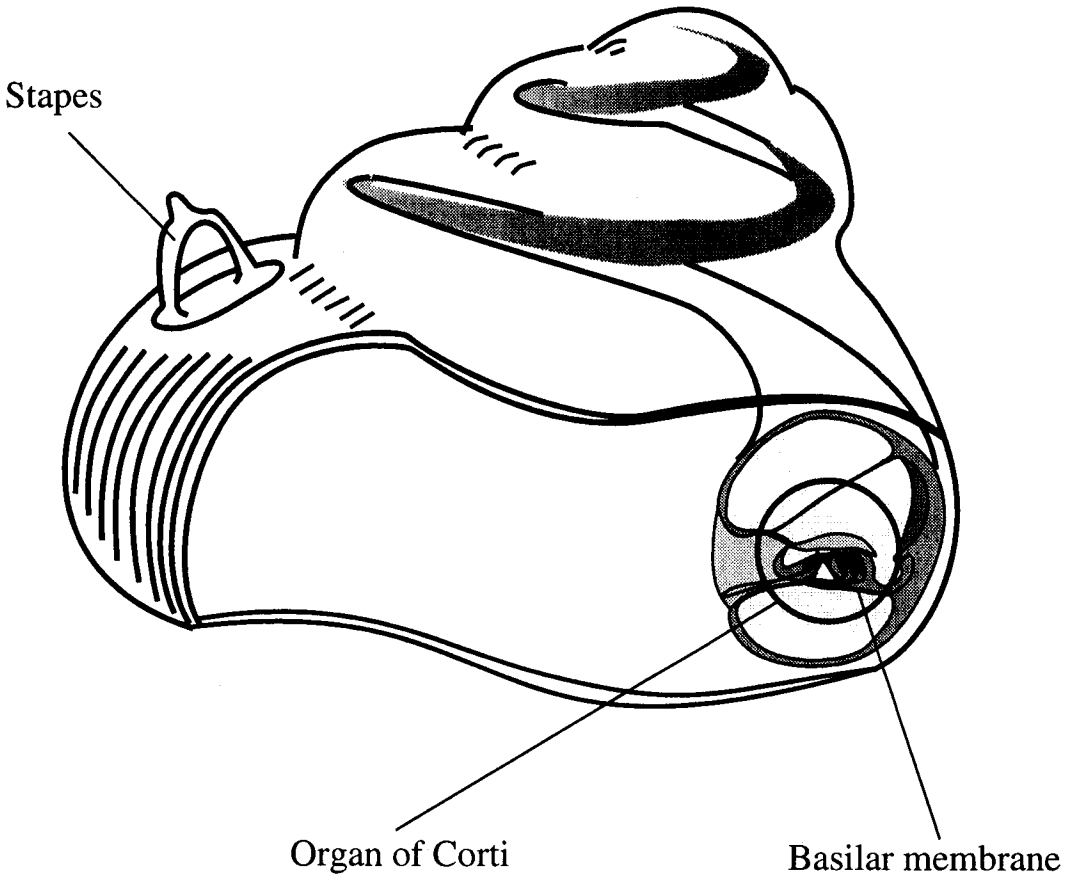


Figure 2.3. Outline of the cochlea of human. The cochlea has triangular shape which is filled with fluid. When the stapes delivers vibrations from the middle war to the fluid-filled cochlea, due to the change in the fluid pressure, the travelling wave occur to the basilar membrane. The basilar membrane supports the sense organ of hearing, the organ of Corti.

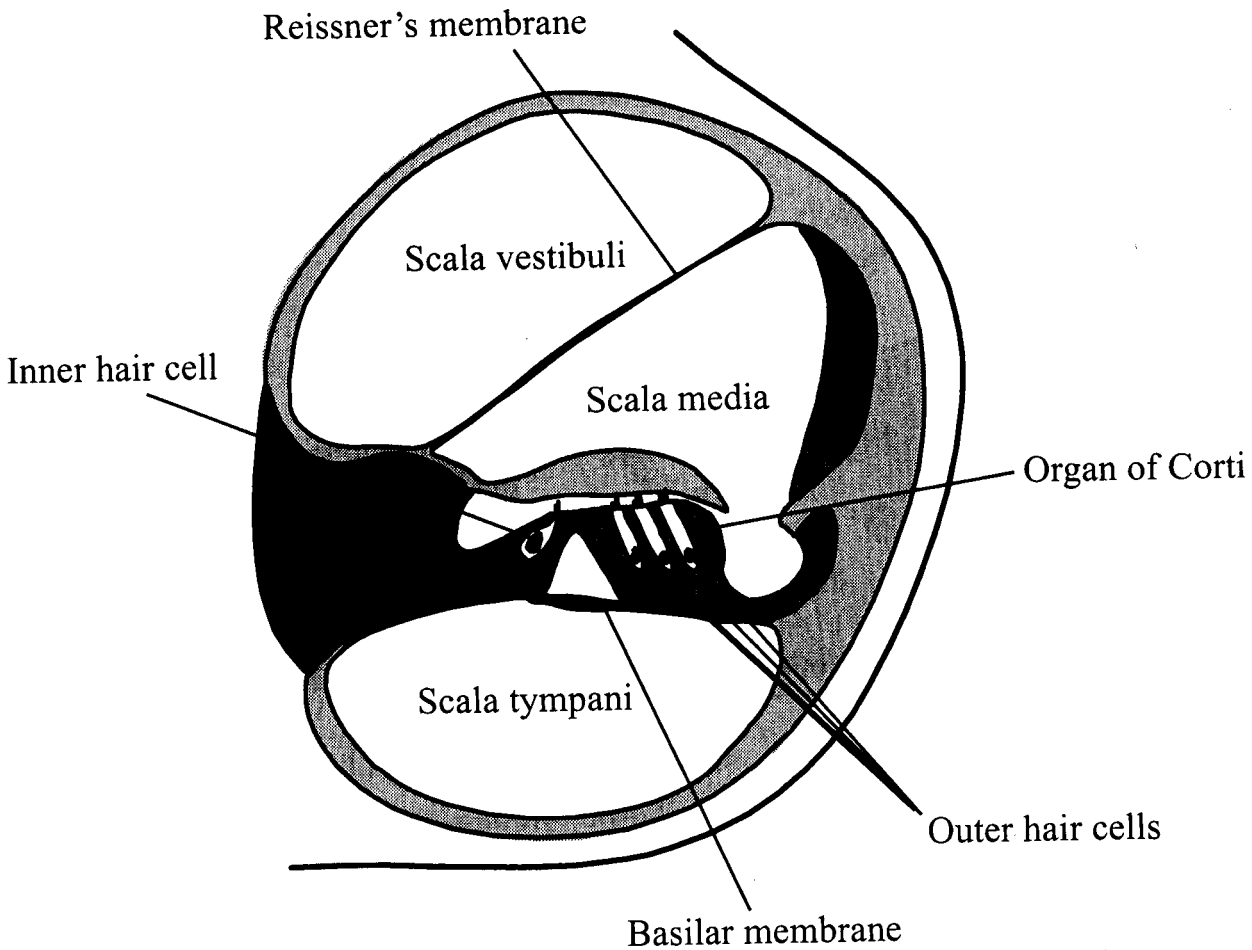


Figure 2.4. Cross section of the cochlea. Cochlear duct is divided into three partitions, scala vestibuli, scala media and scala tympani, and boundaries of the partitions are Ressner's membrane and basilar membrane. The organ of Corti is on the scala media side of the basilar membrane; it contains outer hair cells (OHCs) and inner hair cells (IHCs).

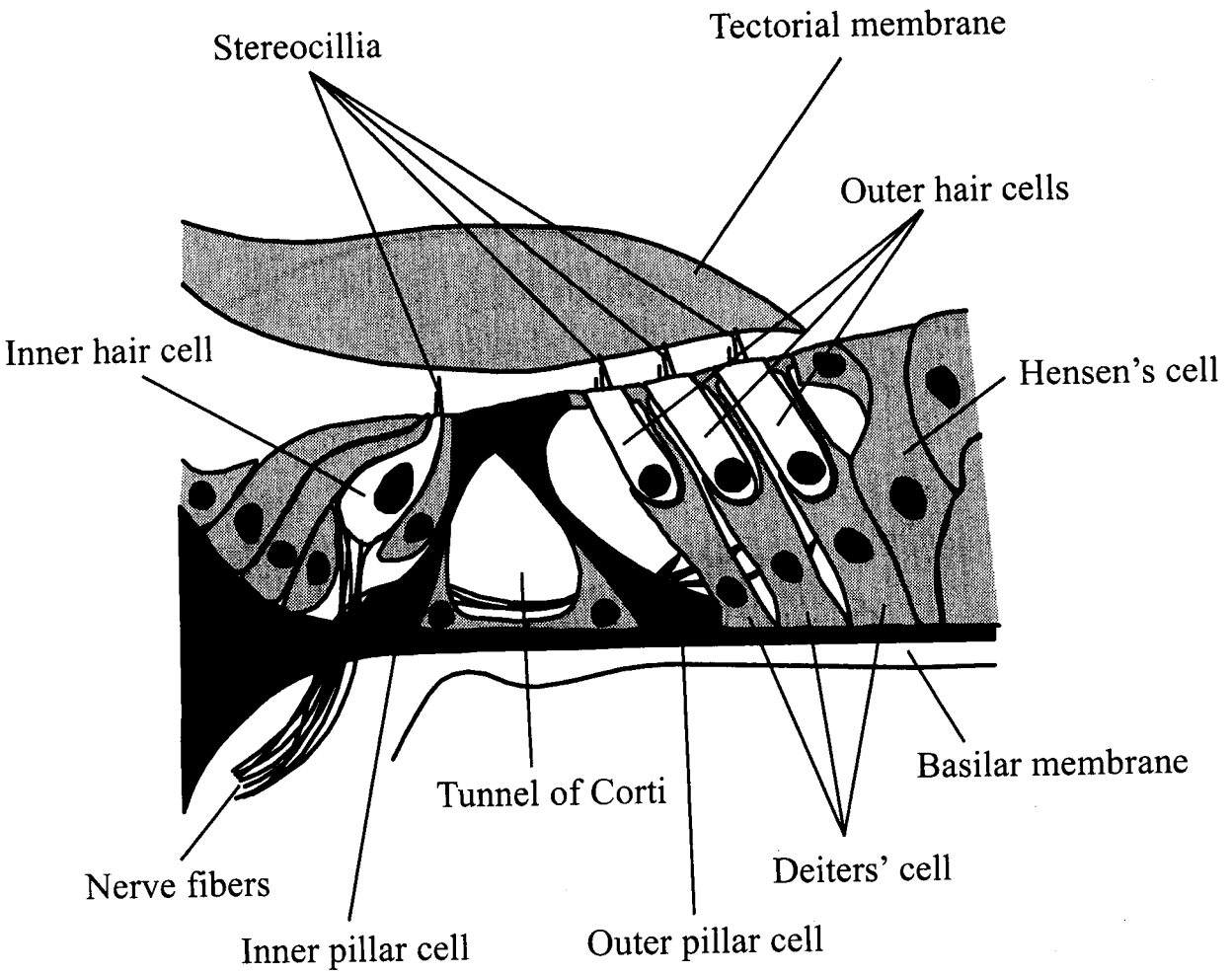


Figure 2.5. Structure of the organ of Corti. The organ of Corti is on the basilar membrane, and it contains an array of supporting cells, outer hair cells and inner hair cells. Tectorial membrane is above the organ of Corti. Nerve fibers enter the organ the central bony core of the cochlea.

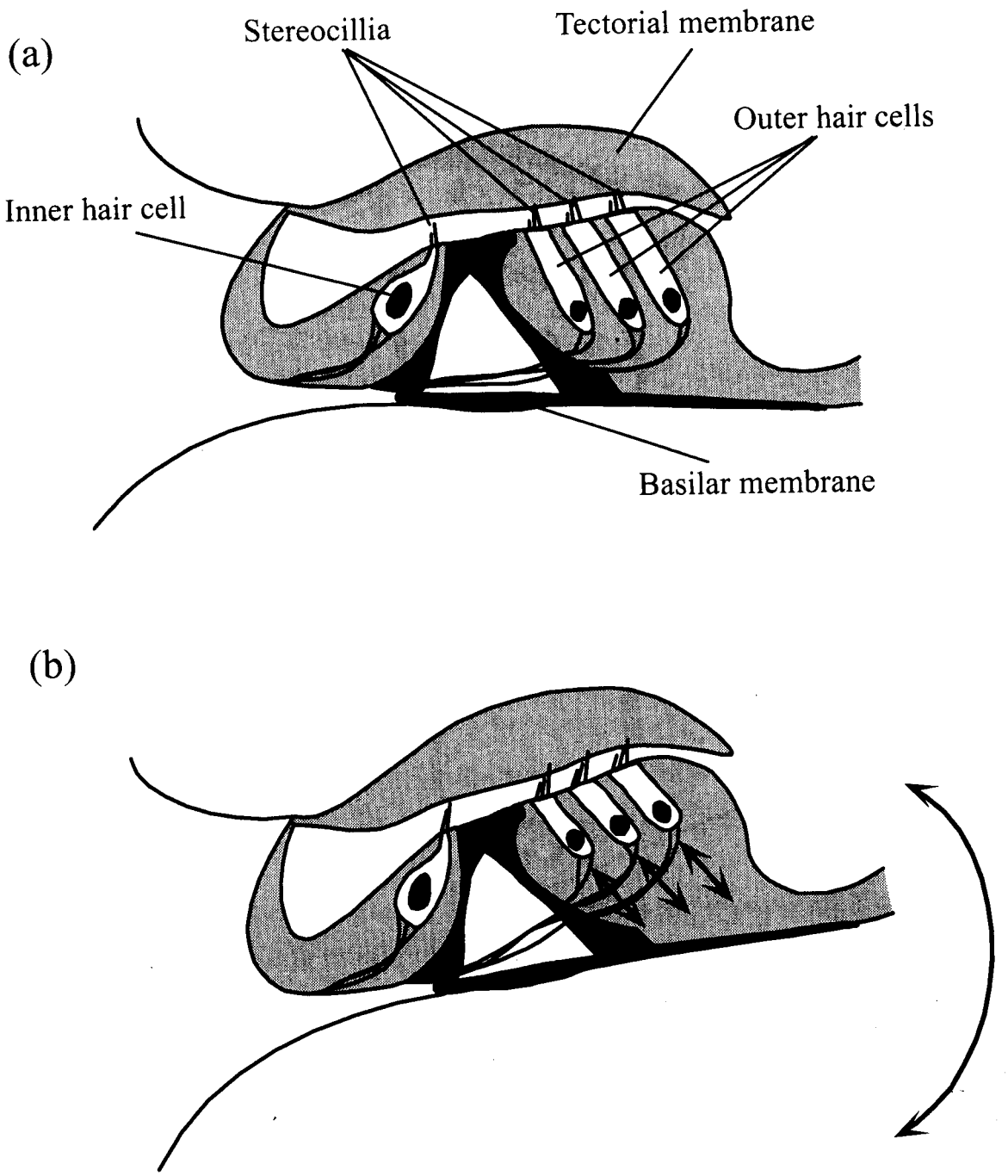


Figure 2.6. Mechanism of the transduction in the organ of Corti. (a) Resting state of the organ of Corti. (b) Shearing motion of the organ of Corti. When the basilar membrane undergoes an oscillatory motion, it produces a relative motion between the reticular lamina and the tectorial membrane. Then, due to the deflection of the stereocillia of the IHC and OHCs, ion flow into the cells and intracellular depolarization causes auditory nerve fiber activation in IHC. Simultaneously, OHCs show motile response and apply forces to the basilar membrane.

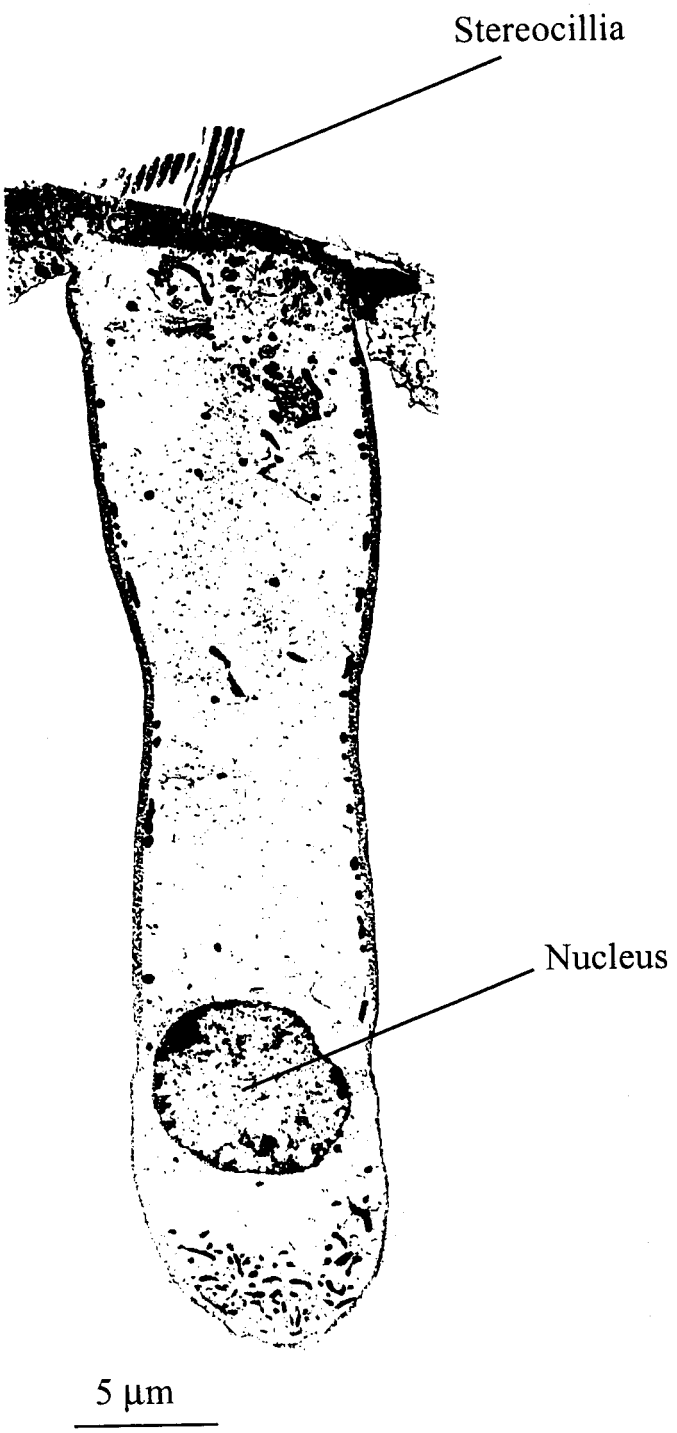


Figure 2.7. Longitudinal section of the outer hair cell which observed using transmission electron microscope (Saito, 1983). Outer hair cell is characteristically cylindrical in shape with a hair bundle, stereocillia, at its apical end and a nucleus near its base. It varies in length from 30 μm to 90 μm, and it has a regular diameter of 10 μm.

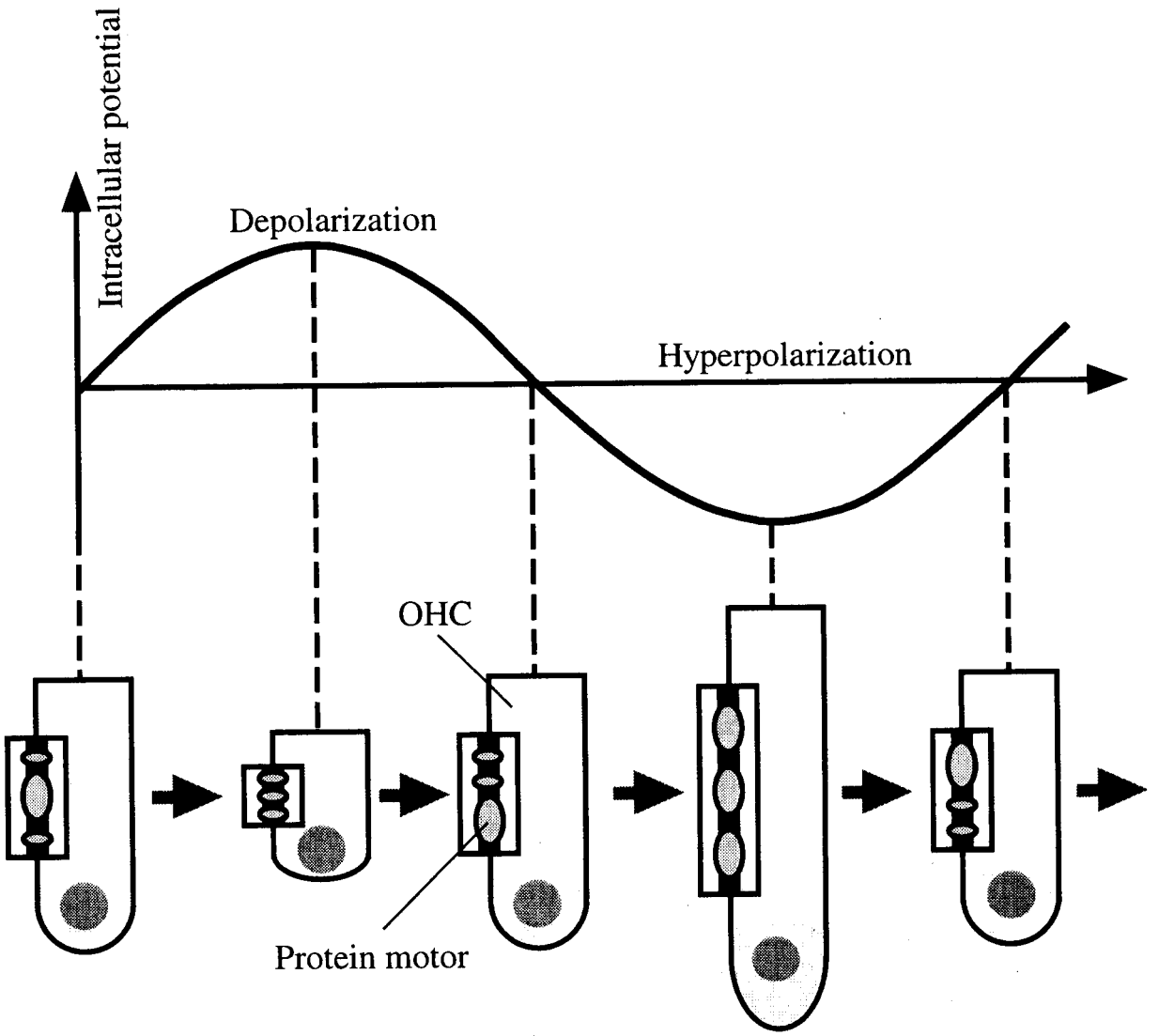


Figure 2.8. Electrically induced length changes of isolated OHC, the so-called electromotility, and its possible molecular mechanisms. In the mammalian cochlea, the plasma membranes of OHCs are replete with intramembrane protein motor. Depolarization causes the proteins to decrease their surface areas within the membrane, causing cellular shortening; hyperpolarization has the opposite effect.

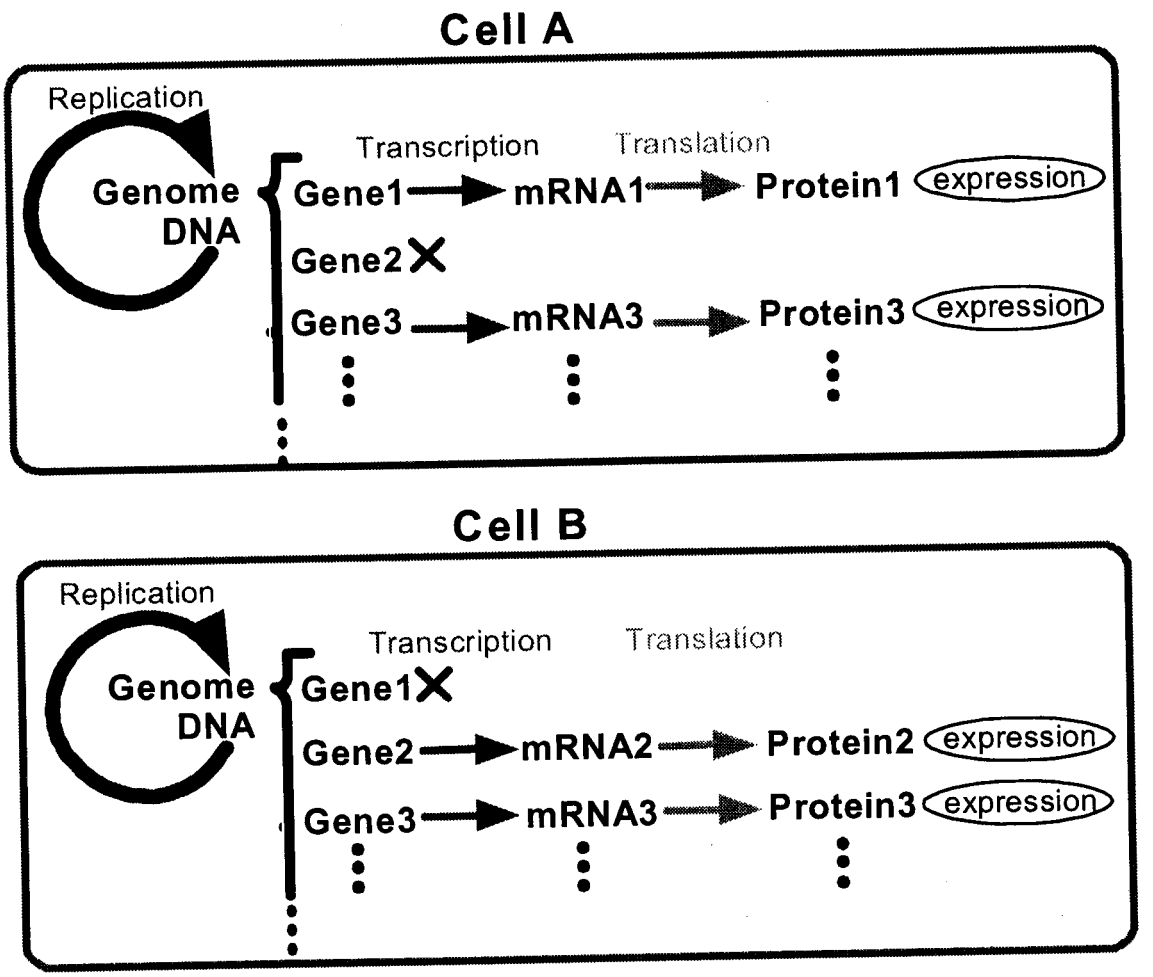


Figure 2.9. The transfer of information from DNA to protein proceeds by means an RNA intermediate called messenger RNA (mRNA). The genes that are important for the function of a particular cell are translated into proteins, so-called gene expression.

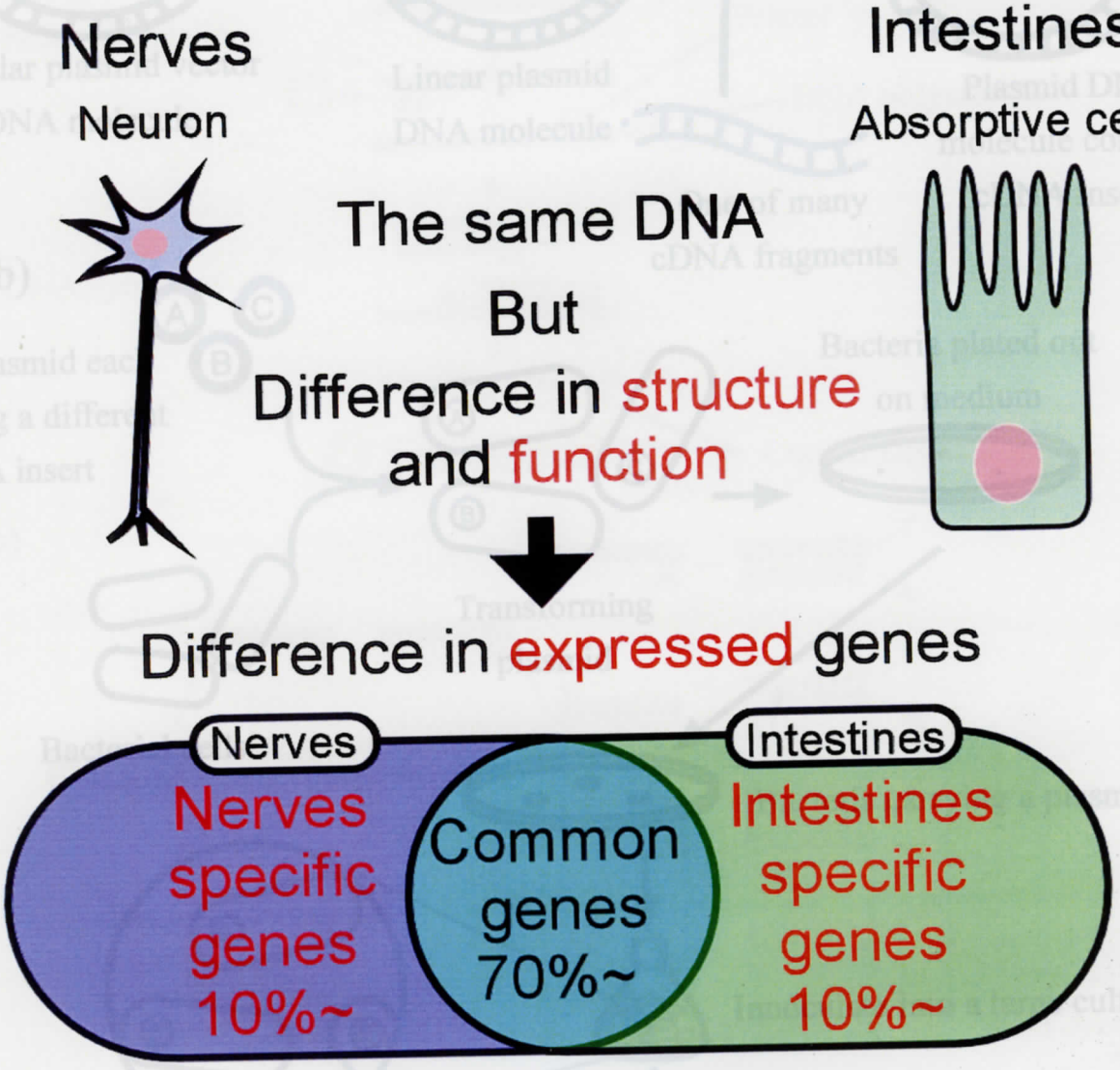


Figure 2.10. The cell types in multicellular organisms become different by expressing different genes from the same genome, although surprisingly few differences in gene expressions distinguish one cell type from another.

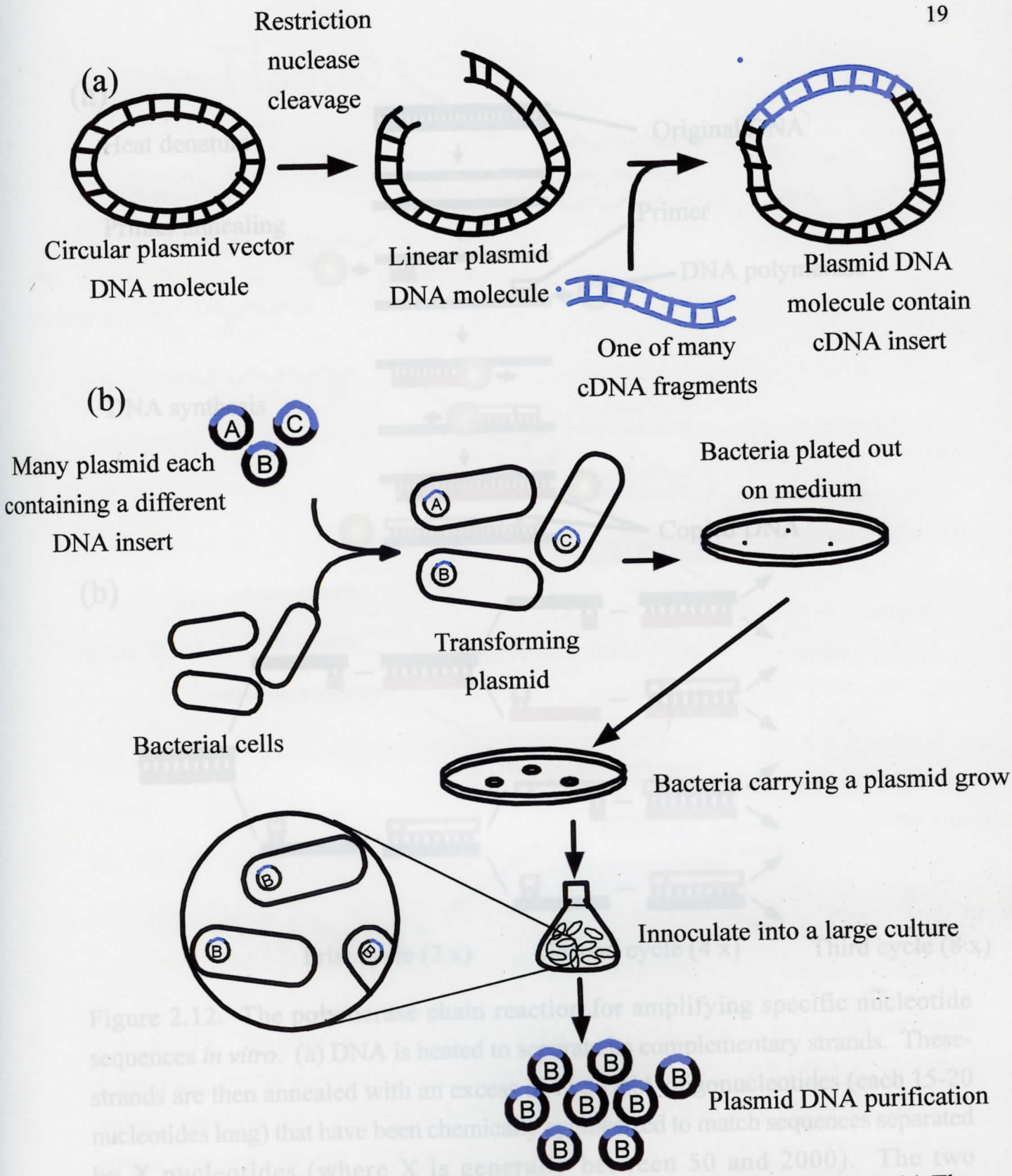


Figure 2.11. The principles underlying the methods used for DNA cloning. (a) The plasmid vector is small circular molecules of double-strand DNA. The plasmid nucleotide ends produced by restriction nuclease, which cut the DNA double helix at specific sequences, allow two DNA fragments to be joined by complementary base-pair interactions. (b) Purification and amplification of a specific DNA sequence by DNA cloning in a bacterium.

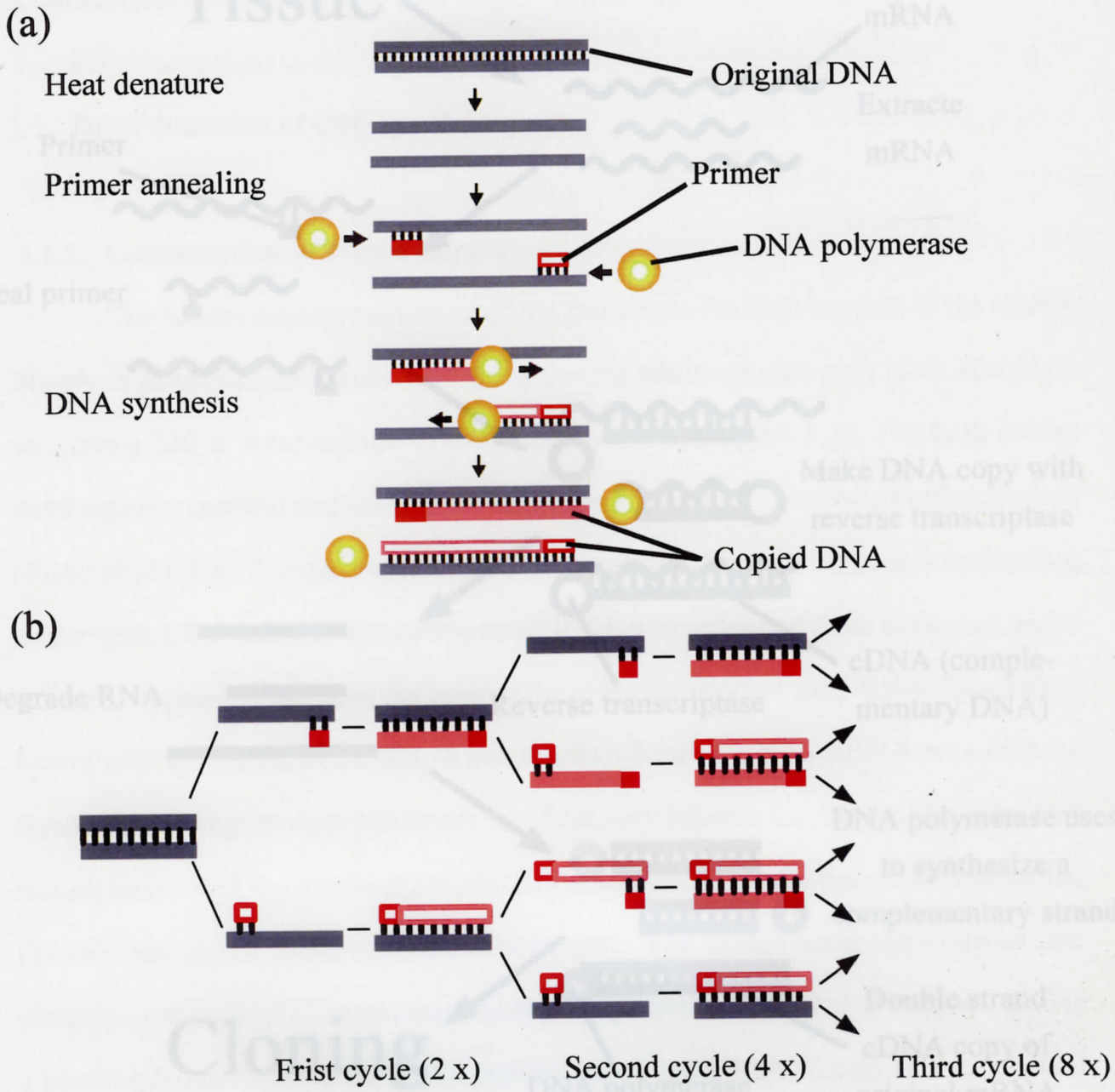


Figure 2.12. The polymerase chain reaction for amplifying specific nucleotide sequences *in vitro*. (a) DNA is heated to separate its complementary strands. These-strands are then annealed with an excess of two DNA oligonucleotides (each 15-20 nucleotides long) that have been chemically synthesized to match sequences separated by X nucleotides (where X is generally between 50 and 2000). The two oligonucleotides serve as specific primers for *in vitro* DNA synthesis catalyzed by DNA polymerase, which copies the DNA between the sequences corresponding to the two oligonucleotides. (b) After multiple cycles of reaction, a large amount of a single DNA fragment, X nucleotides long, is obtained, provided that the original DNA sample contains the DNA sequence that was anticipated when the two oligonucleotides were designed.

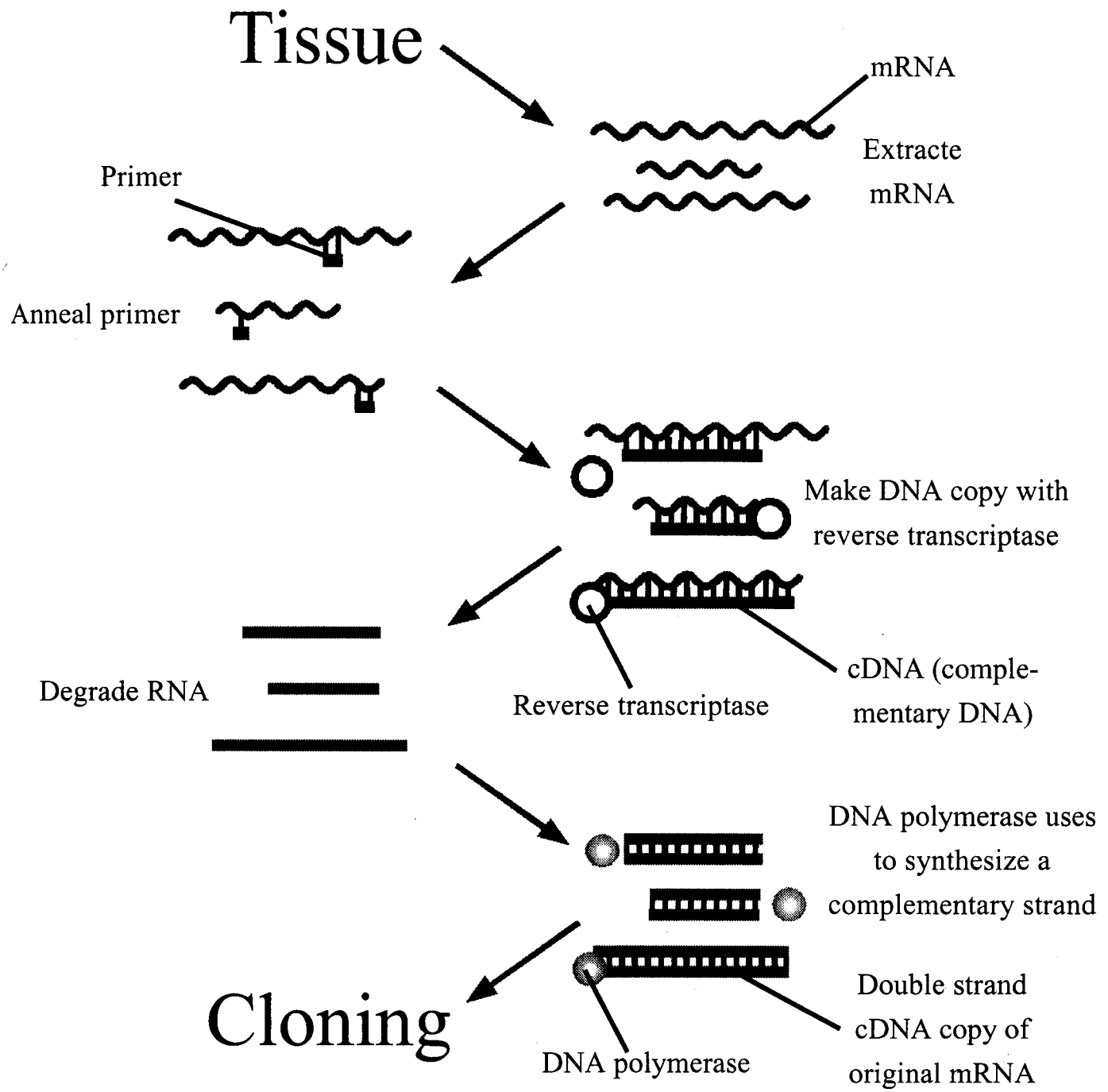


Figure 2.13. The construction of cDNA library. A DNA copy of an mRNA molecule is produced by the enzyme reverse transcriptase, thereby forming a DNA/RNA hybrid helix. Treating the DNA/RNA hybrid with alkari selectively degrades the RNA strand into nucleotides. The remaining single-stranded cDNA is then copied into double-stranded cDNA by the DNA polymerase. As indicated reverse transcriptase require a primer to begin the synthesis. For reverse transcriptase a small oligonucreotides is used; in this example random primer has been annealed with the mRNAs. These cDNAs are inserted into plasmids and cloned (see Figure 2.11). Each clone obtained in this way is called a cDNA clones, and the entire collection of clones derived from one mRNA preparation constitutes a cDNA library.

3. Materials and methods

3.1. Determination of OHC protein motor

3.1.1. Construction of cDNA library

The schematic representation of the procedure for construction of the cDNA library is presented in Figure 3.1. Nine young adults guinea pigs (Std: Hartley), weighting 200 g, were anesthetized by diethyl ether (Figure 3.2). The both bullae were rapidly removed and cochlea was dissected in 0.01 M phosphate buffered saline (PBS) at pH 7.2. The organ of Corti was carefully dissociated from surrounding tissue with a fine needle. Polyadenylated RNA was extracted from dissected organ of Corti, bypassing the need for intermediate purification of total RNA (QuickPrep Micro mRNA Purification Kit, Amersham). Total 1 μg of mRNA was reverse transcribed using random hexamers and Moloney Murine Leukemia Virus reverse transcriptase and the synthesized cDNAs made double-stranded and blunt ended (TimeSaver cDNA Synthesis Kit, Amersham). The cDNA were then ligated into plasmid vector pKF3 (Takara) that had been digested with the Pvu II enzyme, giving a blunt ended defined by the Pvu II cutting site (Figure 3.3). E.coli TH2 competent cells (Takara) introduced to the transforming plasmid according to the manufacture's protocol, and then plated out at a low density to allow for separation of individual clones.

3.1.2. Determination of cDNA insert size

The schematic representation of the procedure for determination of cDNA insert size is presented in Figure 3.4. To evaluate the size of inserts in the cDNA library, inserts were amplified by PCR using pKF3 F1 and R3 primer. PCR

amplification was performed in a 25 μl volume reaction (rTaq DNA Polymerase, Toyobo). We performed 30 cycles of amplification (94 °C for 30 sec, 53 °C for 30 sec, 72 °C for 2 min) using thermal cycler (GeneAmp PCR System 2400, Perkin Elmer Biosystems, Figure 3.5). The samples were then maintained at 4 °C. PCR products were electrophoresed in 2 % agarose gels (Figure 3.6) and visualized by ethidium bromide staining using transilluminator (Gel Print 2000 VGA, Bio Image, Figure 3.6). The clones of no or short insert were excluded from further analysis.

3.1.3. Sequencing and database analysis

The schematic representations of the procedures for sequencing and database analysis is presented in Figure 3.7 and 3.8. The clones, of which cDNA was longer than approximately 200 bp, were selected and the plasmid DNAs were recovered using GFX Micro Plasmid Prep Kit (Amersham). Selected clones were sequenced by the standard deoxynucleotide sequencing system (ALFexpress DNA Sequencer, Amersham, Figure 3.9) using pKF3 F1 and R4 primer. Sequence analysis was performed using the GENETYX-MAC ver.10.1 software (Software Development). After the redundant clones were removed, nucleotide sequences of clones were compared to the release 40 of the DDBJ human, primate, mammalian, rodent and vertebrate databases using the FASTA 3.0 program. The clones were then classified as known, unknown genes and contaminations according to their match rate for known sequences deposited in the DDBJ databases.

3.1.4. Tissue expression analysis using RT-PCR

Clones showing no significant match with known sequences deposited in the DDBJ databases were selected. The each PCR primer of the selected clones were designed using the utility of GENETYX-MAC ver.10.1 software. Based on the

designed primers, more optimum clones to perform the PCR analysis were chosen. The tissue expressions of chosen clones were assessed by the sensitive RT-PCR (Rverse Transcription PCR) approach. The guinea pig mRNAs from 10 different tissues (cochlea, cerebellum, kidney, liver, heart, brain, spleen, lung, eye and testis) were isolated as described above. One micrograms of mRNA was reverse-transcribed in the presence of random primers according to the manufacturer's instructions (First-Strand cDNA Synthesis Kit, Amersham) and 1/20 of each sample was used as template for PCR amplification using specific primers for each clone or G3PDH primers. Thirty cycles of PCR (94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 1min) were performed. PCR products were electrophoresed in ethidium bromide 2 % agarose gel.

3.2. Cloning the gerbil prestin cDNA

3.2.1. First-strand cDNA synthesis

The schematic representation of the procedure for cloning is presented in Figure 3.11. Seven adult gerbils (Figure 3.12) were anesthetized by diethyl ether. The bullae were rapidly removed and cochleae were dissected. Polyadenylated RNA was extracted from the dissected cochleae, bypassing the need for intermediate purification of total RNA (QuickPrep *Micro* mRNA Purification Kit; Amersham Pharmacia Biotech, Buckinghamshire, UK). Reverse transcription was performed from the polyadenylated RNA using random hexamers and Moloney Murine Leukemia virus reverse transcriptase (First-Strand cDNA Synthesis Kit; Amersham Pharmacia Biotech).

3.2.2. PCR amplification

To obtain the full length coding sequence of prestin cDNA, eight primers were designed (Table 3.1) in order that amplified fragments by these primers include the same sequence (10bp or so) in each termini. PCR amplification was performed in a 50μl volume reaction with a thermostable DNA polymerase (KOD -Plus-; Toyobo, Osaka, Japan). We performed 35 cycles of amplification (94°C for 15 sec, 51°C for 30 sec, 68°C for 45 sec) using a thermal cycler (GeneAmp PCR system 2400; PE Biosystems, Figure 3.5). The samples were then maintained at 4 °C. These PCR products were electrophoresed in 1.5% agarose gels. Obtained four fragments were called pres1, pres2, pres3, and pres4, respectively.

3.2.3. Cloning and sequencing

The schematic representation of the procedure for cloning is presented in Figure 3.13. These four PCR products were then ligated into a plasmid vector pCR-Blunt II- TOPO (Invitrogen, CA, Figure 3.14). The *E. coli* TOP10 competent cells (Invitrogen) were transformed by the plasmid according to the manufacture's protocol, and then plated out. The colonies were picked and cultured. Then, the plasmid DNAs were purified using GFX Micro Plasmid Prep Kit (Amersham Pharmacia Biotech). To estimate the length of the inserts, the plasmid was digested with restriction enzyme EcoR I and the products were electrophoresed in 1.5% agarose gels. The positive clones were sequenced bidirectionally by fluorescence-coupled autosequencing system (ALFexpress DNA Sequencer; Amersham Pharmacia Biotech, Figure 3.9) using a sequencing kit (ALFexpress AutoRead Sequencing Kit; Amersham Pharmacia Biotech). The obtained data were analyzed using GENETYX-MAC ver.10.1 software (Software Development, Tokyo, Japan). Then, the inserted cDNA corresponding to the prestin sequence was directly amplified by PCR.

3.2.4. Combining adjacent fragments

The adjacent fragments were combined in the procedure shown in Figure 3.15. First, the adjacent fragments were heated to separate these complementary strands. These strands were then annealed with an adjacent strand because these two strands contained the same sequence. As the annealing site served as a primer, KOD-Plus- DNA polymerase synthesized the DNA downstream from this site. Next, the combined fragment was amplified by PCR. Finally, a large number of combined fragments were obtained.

According to this procedure in Figure 2.8, first, the pres1 fragment was combined with the pres2 fragment and the pres3 fragment was combined with the pres4 fragment, respectively under the following condition : denature step at 94°C for 15 sec, annealing step at 51°C for 30 sec, and extension step at 68°C for 45 sec. These combined fragments were called pres1&2 and pres3&4. 30 cycles of PCR were carried out for the pres1&2 and the pres3&4 fragments using PRES-F185 and PRES-R1420 primers (pres1&2), PRES-F1410 and PRES-R2570 primers (pres3&4) under following condition : denature step at 94°C for 15 sec, annealing step at 51°C for 30 sec, and extension step at 68°C for 1 min 15 sec. Then, pres1&2 and pres3&4 were subcloned. The positive clones of pres1&2 and pres3&4 were directly amplified by PCR. The same procedure was done for combining the pres1&2 fragment with the pres3&4 fragment under the following condition : denature step at 94°C for 15 sec, annealing step at 55°C for 30 sec, and extension step at 68°C for 1 min 20 sec. The combined fragment was amplified 30 cycles of PCR (94°C for 15 sec, 68°C for 2 min 30 sec) using PRES-F185 and PRES-R2570 primers. Finally, the complete length of prestin cDNA was obtained and subcloned for sequencing. Sequencing was carried out using BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (sApplied Biosystems, Foster City, CA) and an automated DNA sequencer (ABI

Prism 310; Applied Biosystems, Figure 3.16).

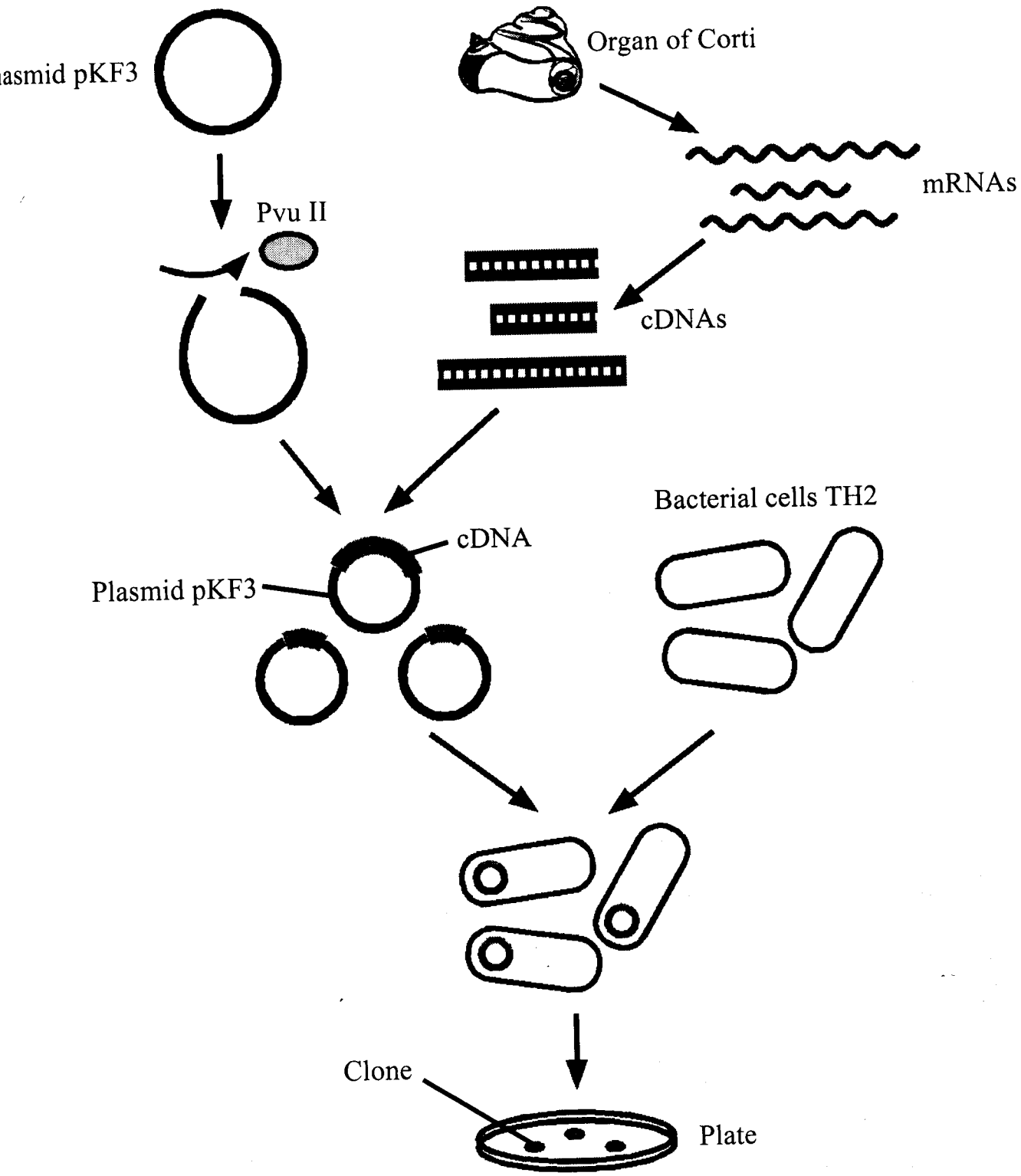


Figure 3.1. The method of the cDNA library construction in this study.

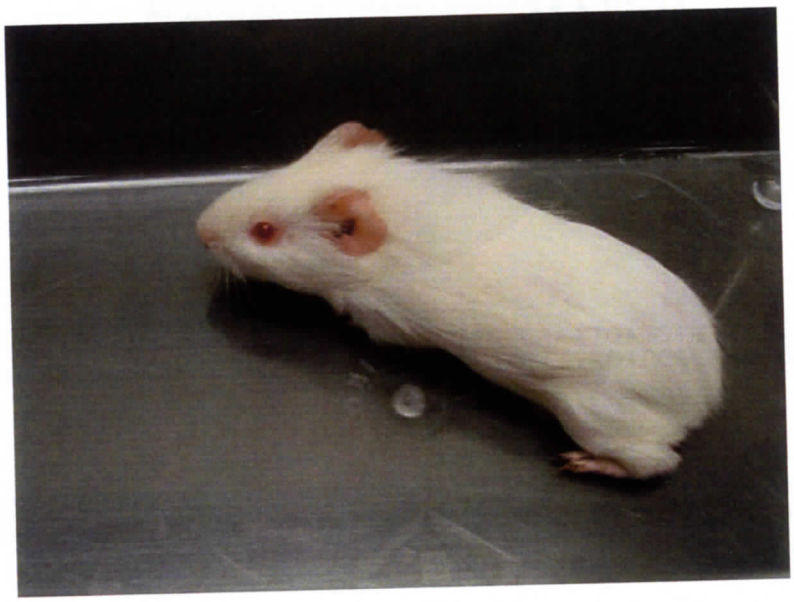


Figure 3.2. An appearance of sacrificed guinea pig (Std: Hartley) in this study.

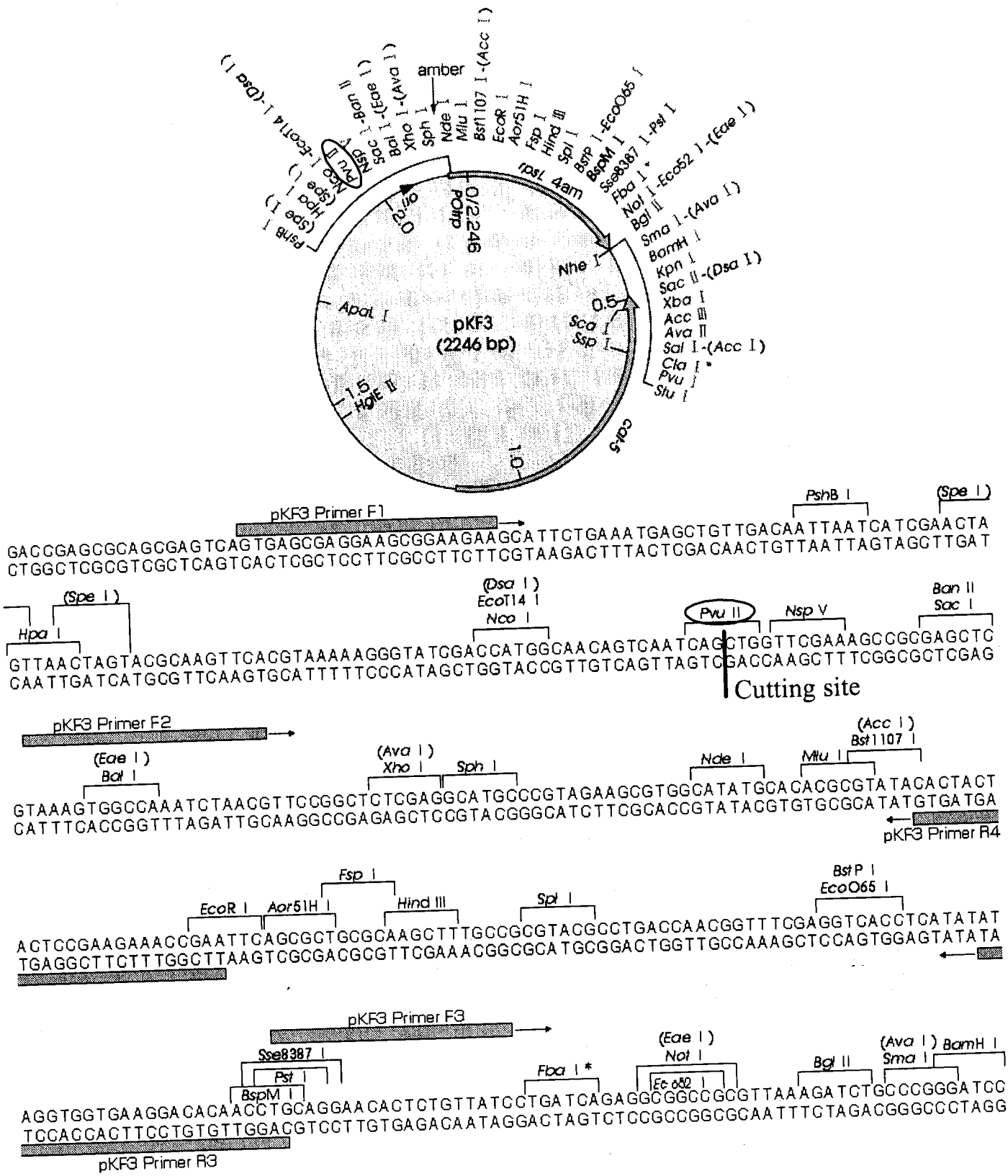


Figure 3.3. Partly DNA sequence of plasmid vector pKF3. The cDNAs were ligated into pKF3 that had been digested with the Pvu II cutting site.

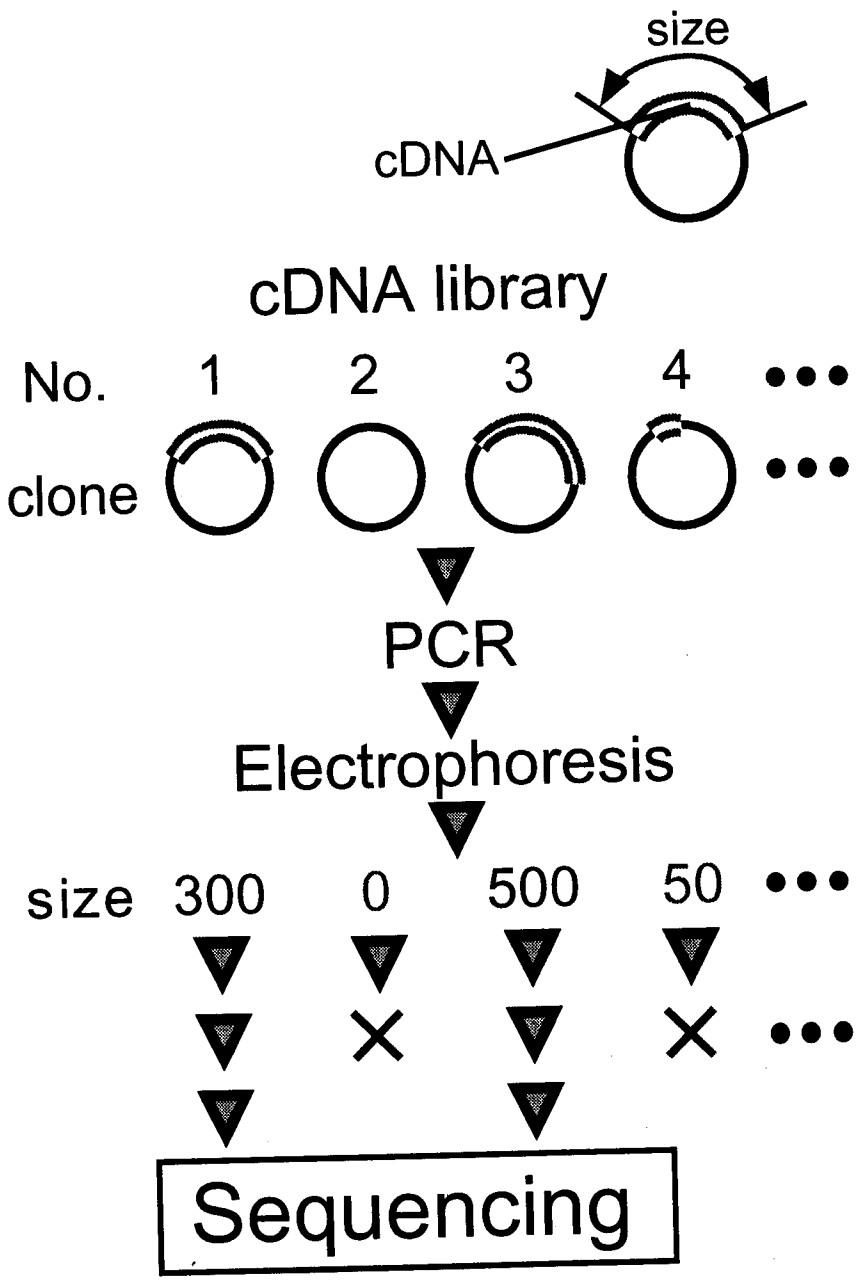


Figure 3.4. Determination of cDNA insert size. To evaluate the size of inserts in the cDNA library, inserts were amplified by PCR, and were electrophoresed. The clones of no or short insert were excluded from further analysis.

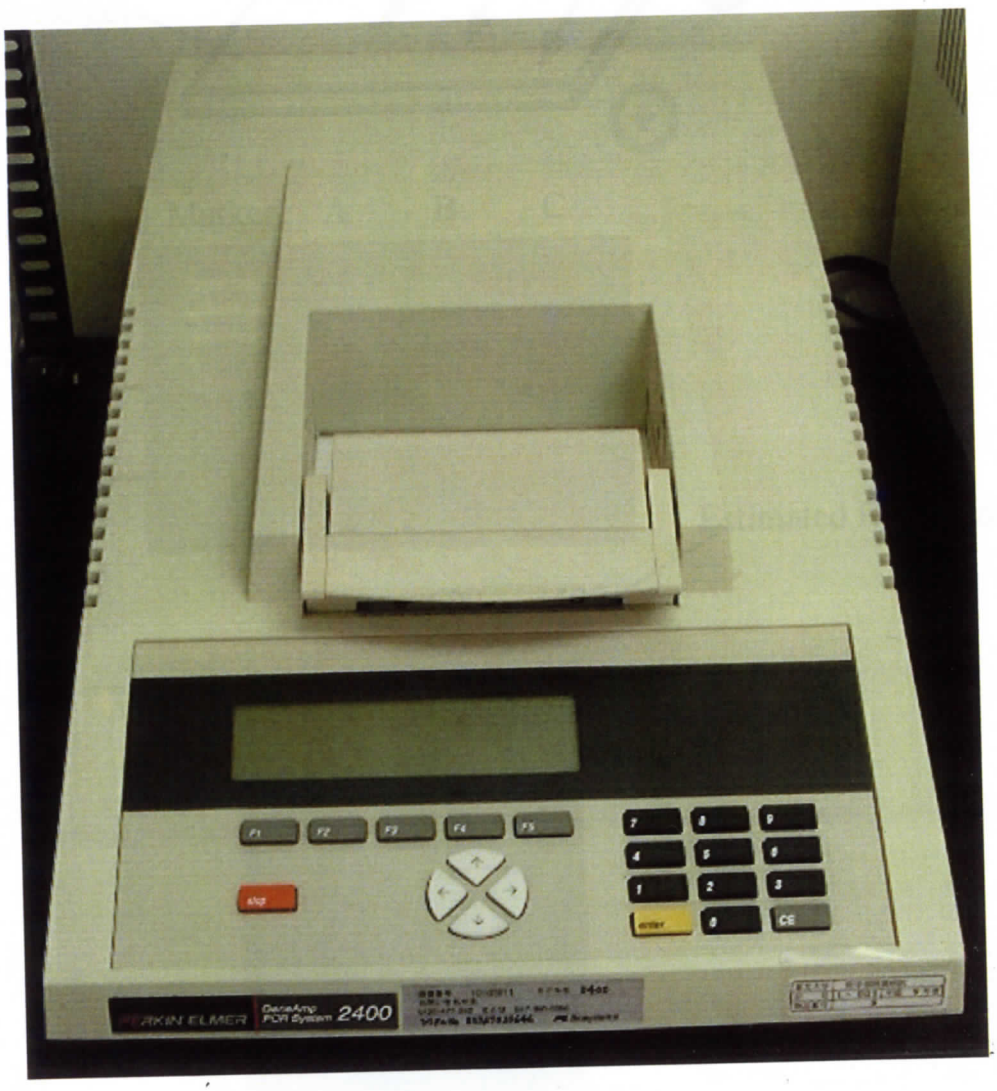


Figure 3.5. Thermal cycler (GeneAmp PCR System 2400; Perkin Elmer Biosystems).

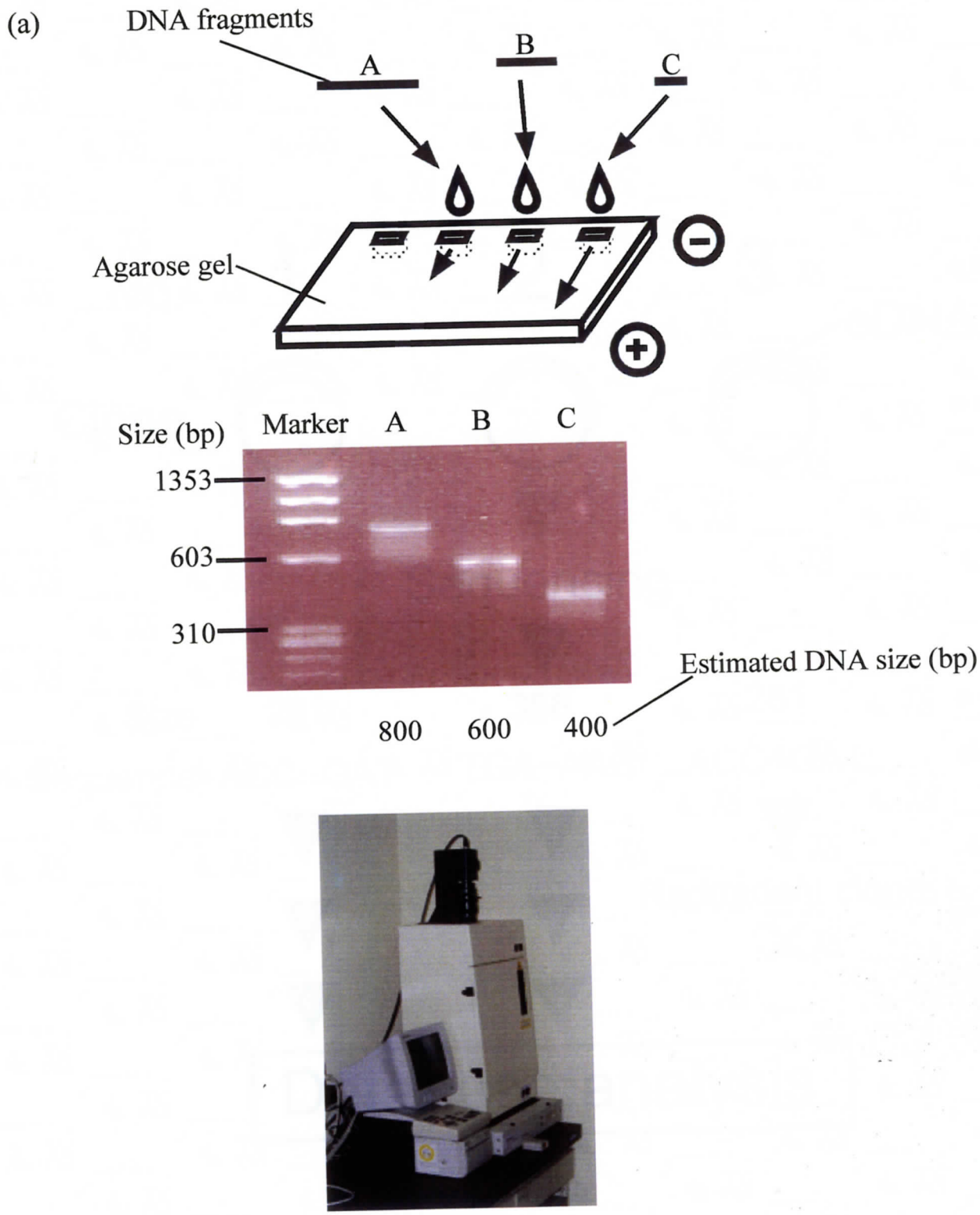


Figure 3.6. (a) Gel electrophoresis is a powerful technique for determining size of DNA molecule. Each DNA carries a single negative charge. If an electric field is applied, the DNA molecules move to anode at the speed according to thier size. The molecules can be detected by their fluorescence when stained with the dye ethidium bromide. (b) Transilluminator (Gel Print 2000 VGA, Bio Image).

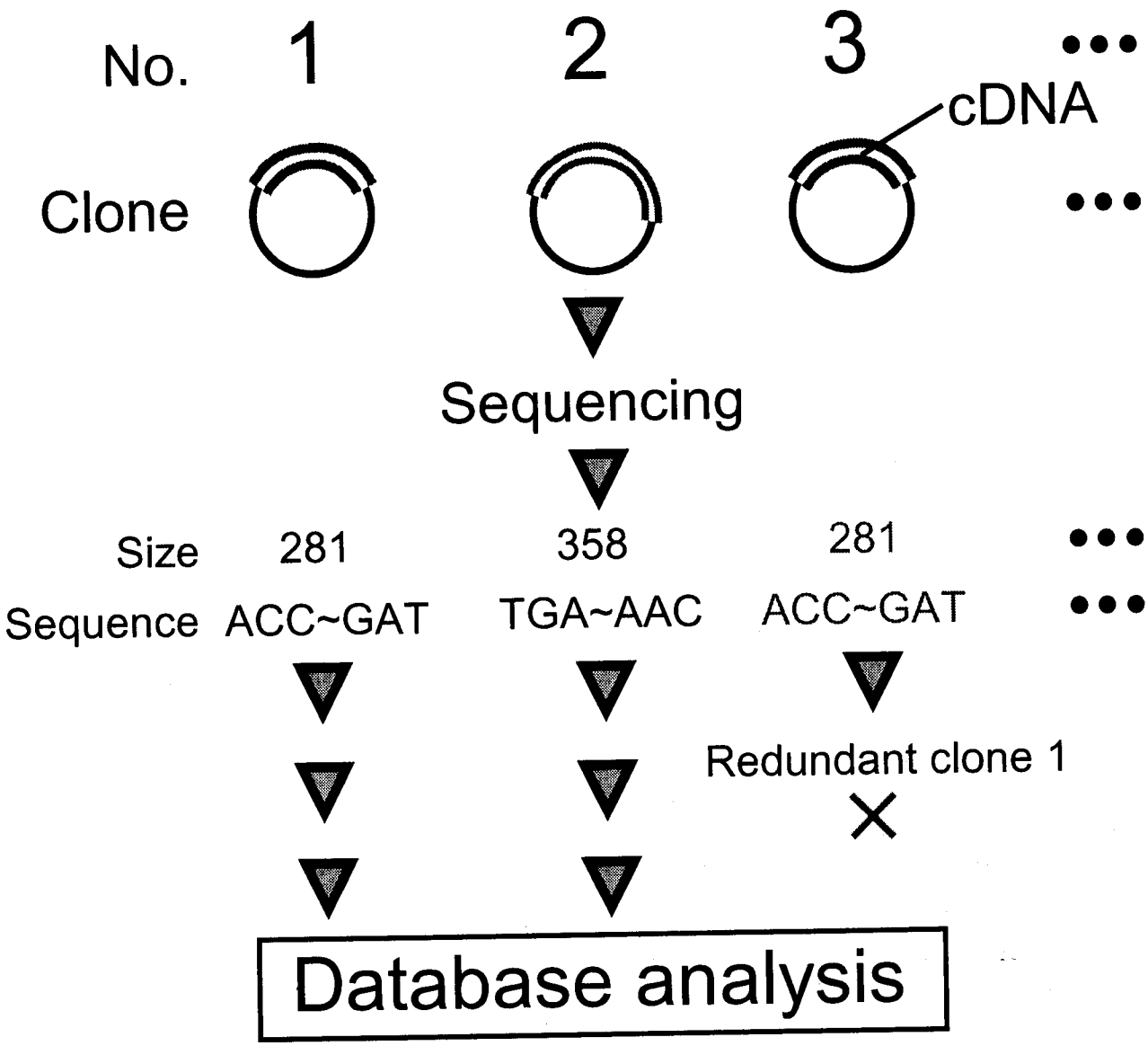


Figure 3.7. Sequencing. Selected clones were sequenced, and redundant clones were removed.

(a)

(b)

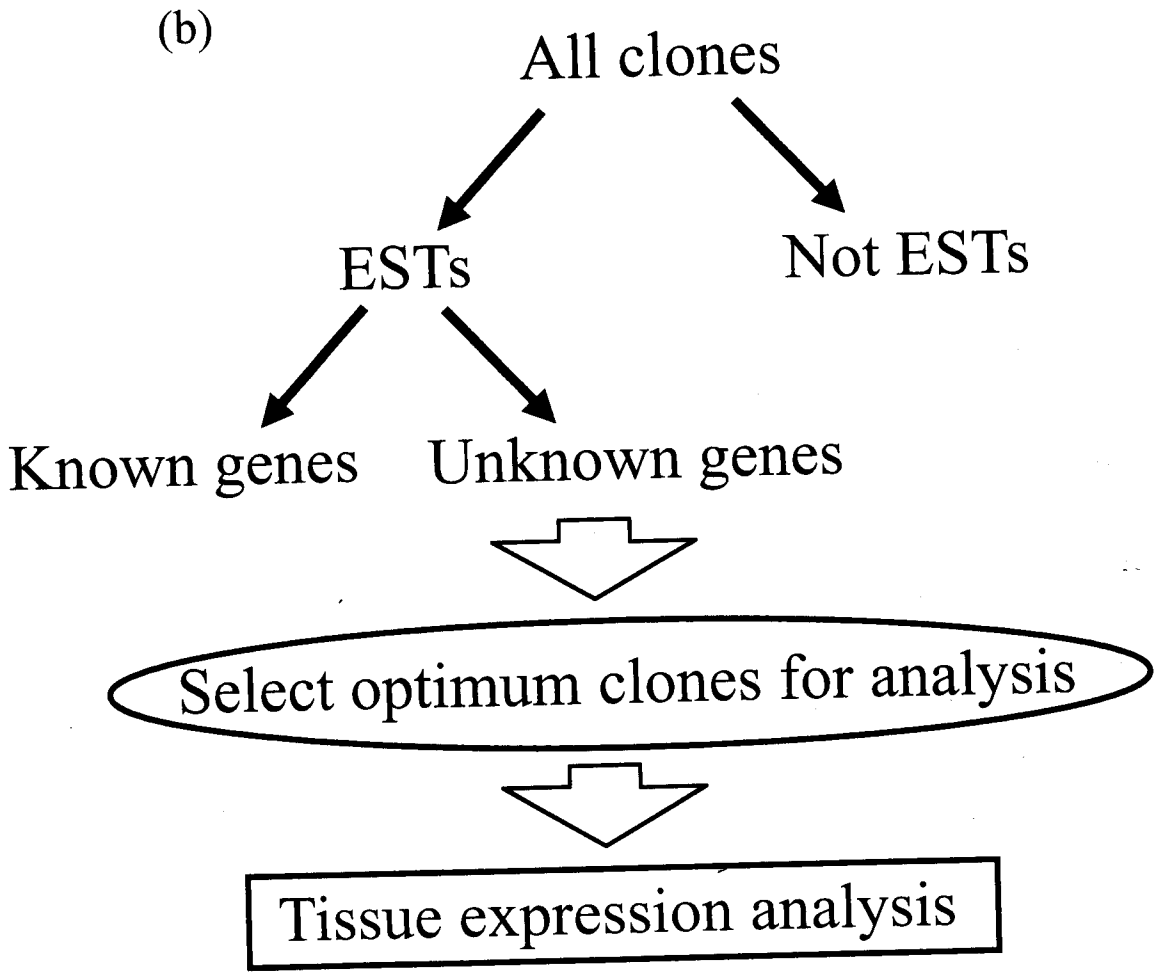
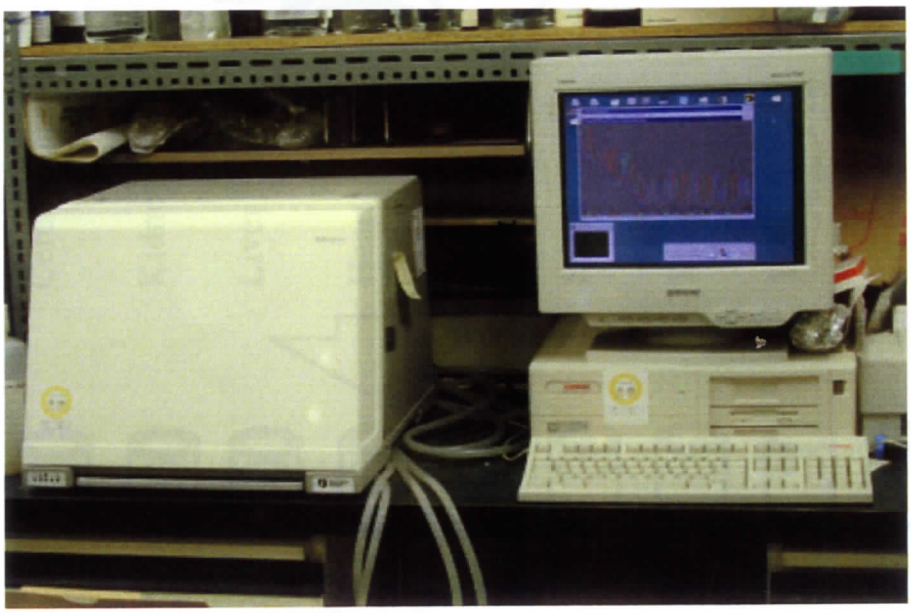


Figure 3.8. Database analysis. (a) DDBJ homepage. (b) Distribution of clones. The clones were classified into known, unknown genes and contaminations according to their match rate for known sequences deposited in the DDBJ databases.

(a)



(b)

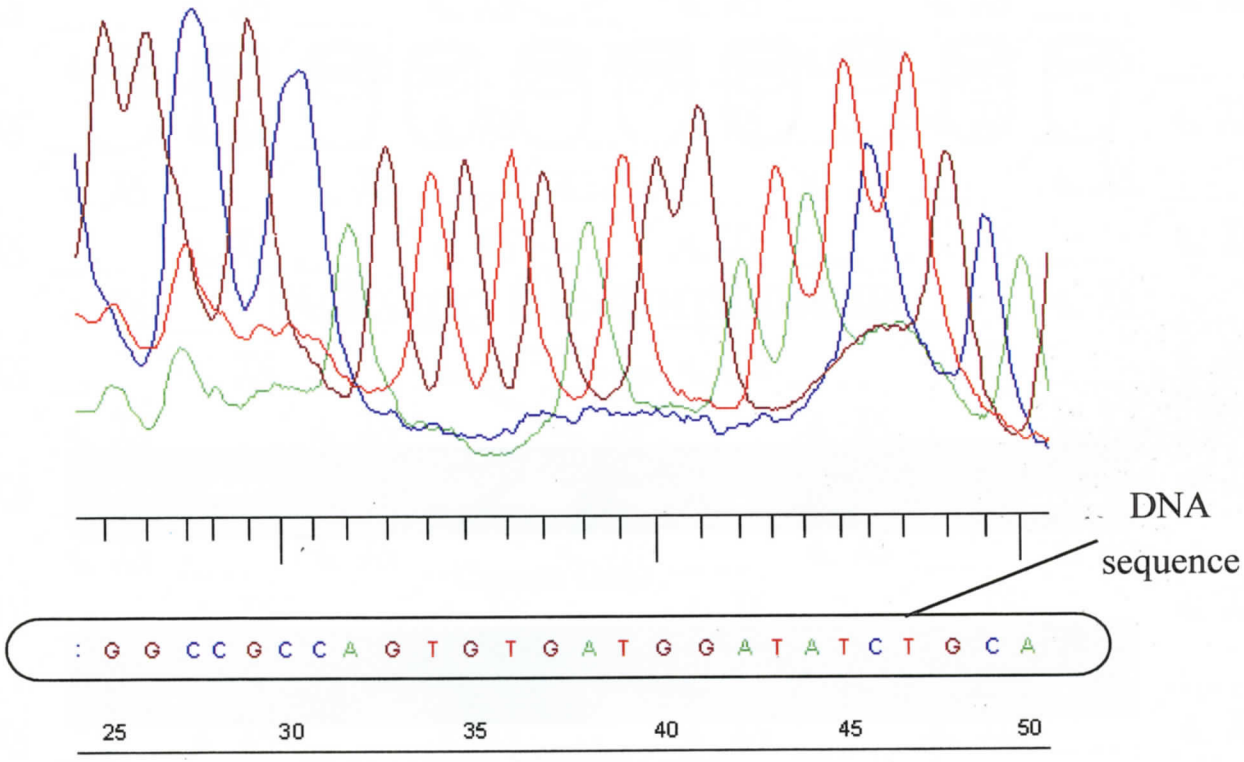


Figure 3.9. (a) Deoxynucleotide sequencing system (ALFexpress DNA Sequencer, Amersham). (b) An example of its output result.

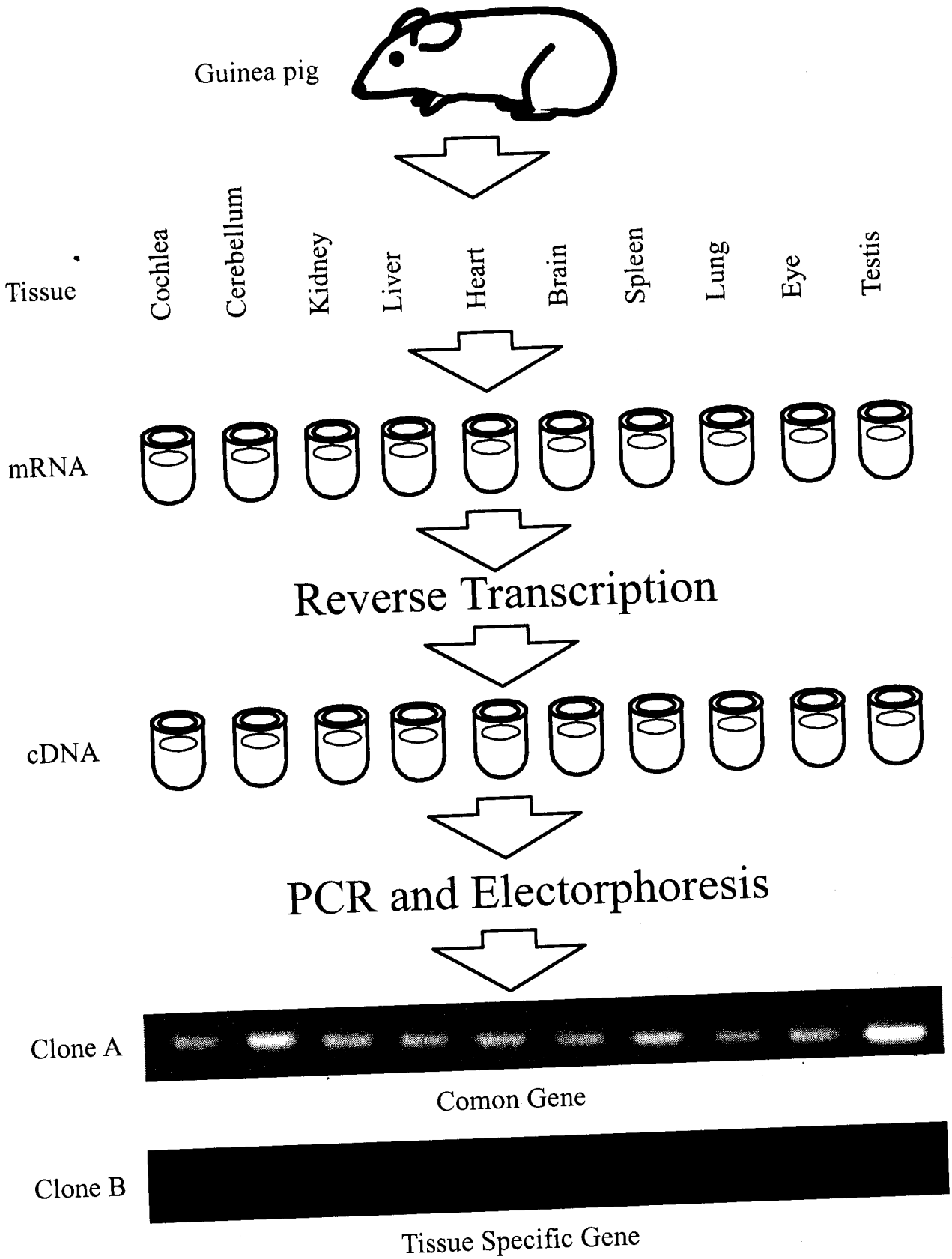


Figure 3.10. Tissue expression analysis using RT-PCR. Clones showing no significant match with known genes were selected and more optimum clones to perform the PCR analysis were chosen. Their tissue expressions were assessed by the sensitive RT-PCR approach.

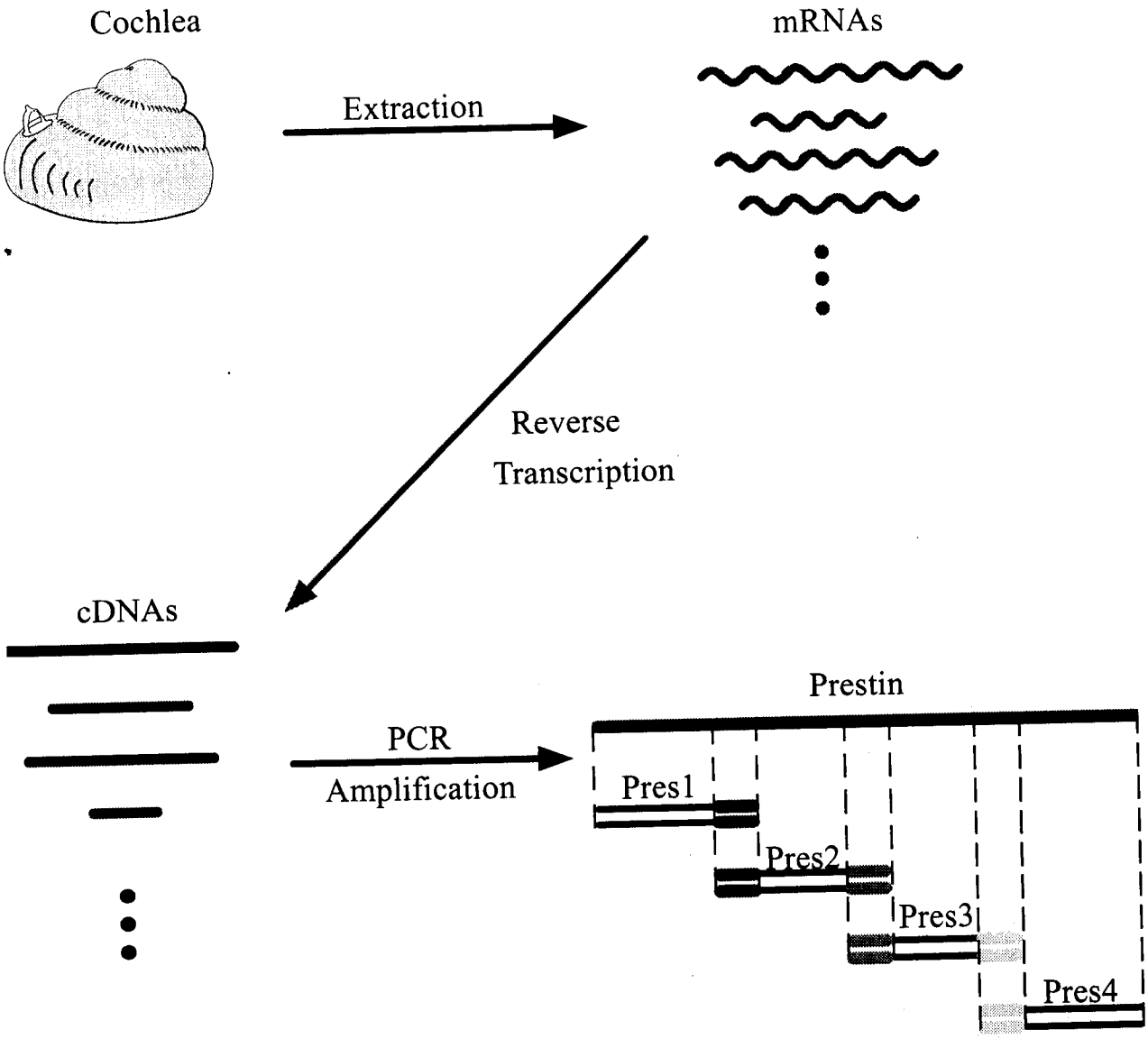


Figure 3.11. The method of cloning in this study. Polyadenylated RNA was extracted from seven adult cochleae. The mRNA was reverse transcribed in the presence of random hexanucleotides. PCR was performed with the gerbil cochlear cDNA and the synthesized primers, and obtained four fragments were called pres1, pres2, pres3, and pres4, respectively.

Figure 3.12. A gerbil. Seven mature gerbils were anesthetized and cochleae were rapidly removed.

Table 3.1. The sequence of primers for gerbil prestin.

Primer name	Sequence	Length(bp)
Pres1		
PRES-F185	5'-AAATGCTCGTCTCCTGCTGTTGGTGAAT-3'	28
PRES-R829	5'-GGCCACAAATCCAAACCTGCAC-3'	22
Pres2		
PRES-F808	5'-GTGCAGGTTTGGATTTGTGGCC-3'	22
PRES-R1420	5'-GCGAGACAAGGAGCAGGAAATG-3'	22
Pres3		
PRES-F1410	5'-CCTTGTCTCGCAGCCTTGTTCA-3'	22
PRES-R1958	5'-CGTACTTCCTCATGGCCTTCCT-3'	22
Pres4		
PRES-F1945	5'-CATGAGGAAGTACGCAAAGGAA-3'	22
PRES-R2570	5'-ACTACTAACATTTTCCTTGGGGGTTGGG-3'	28

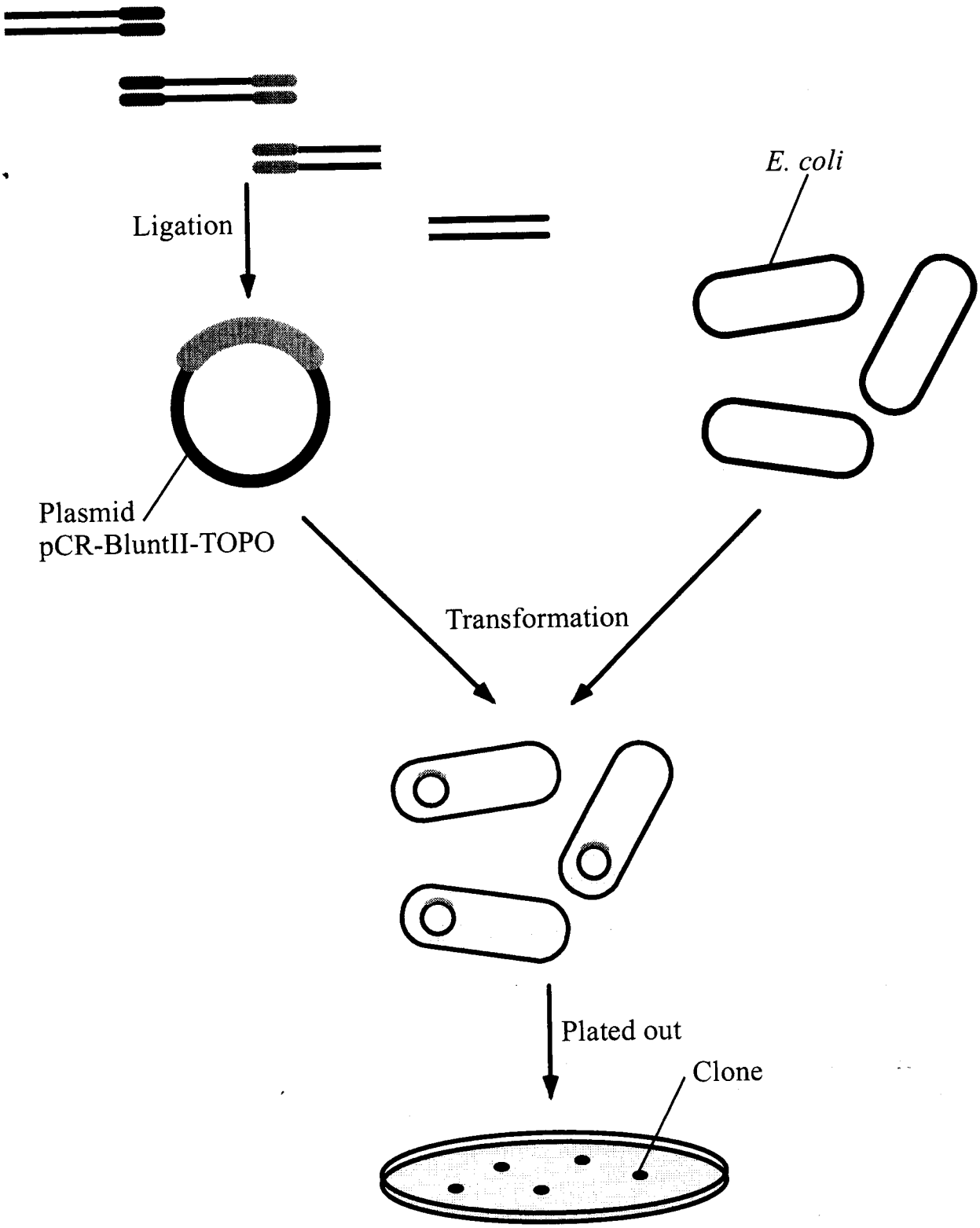


Figure 3.13. Ligation and Transformation. The four PCR product were ligated into a plasmid vector. These resulting plasmid were then transfected into *E. coli.* and plated out.

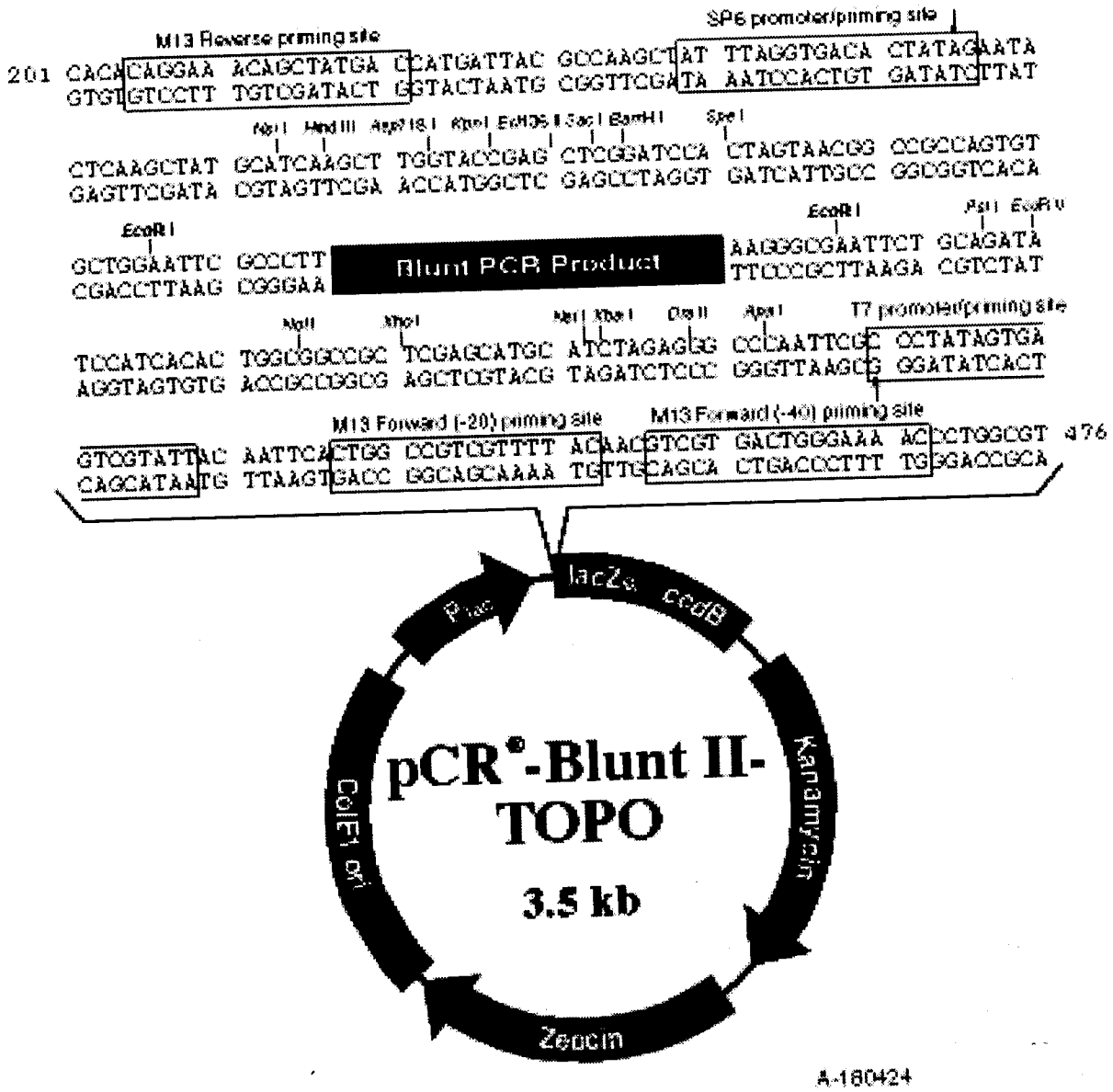


Figure 3.14. Partly DNA sequence of plasmid vector pCR-Blunt II-TOPO (Invitrogen).

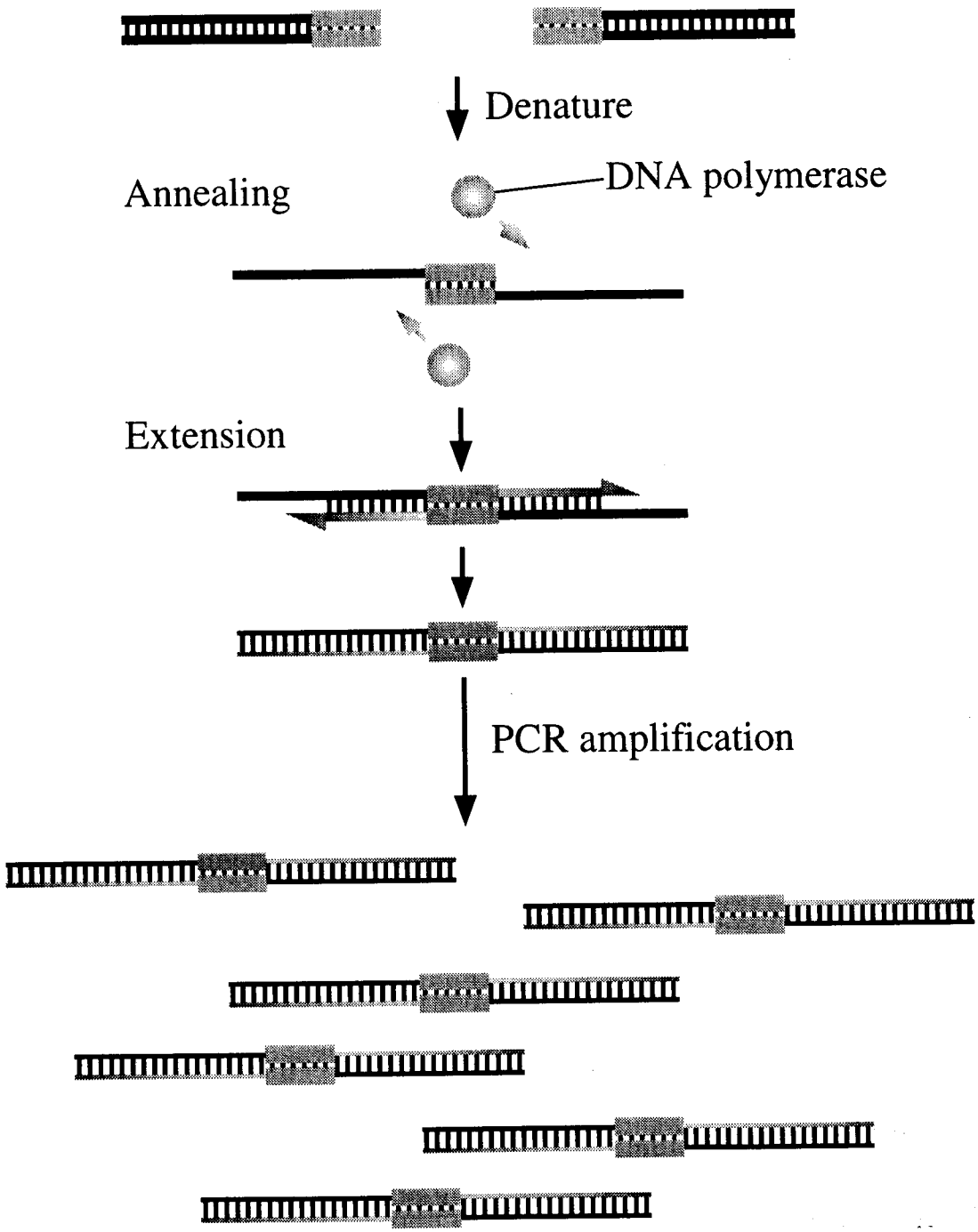
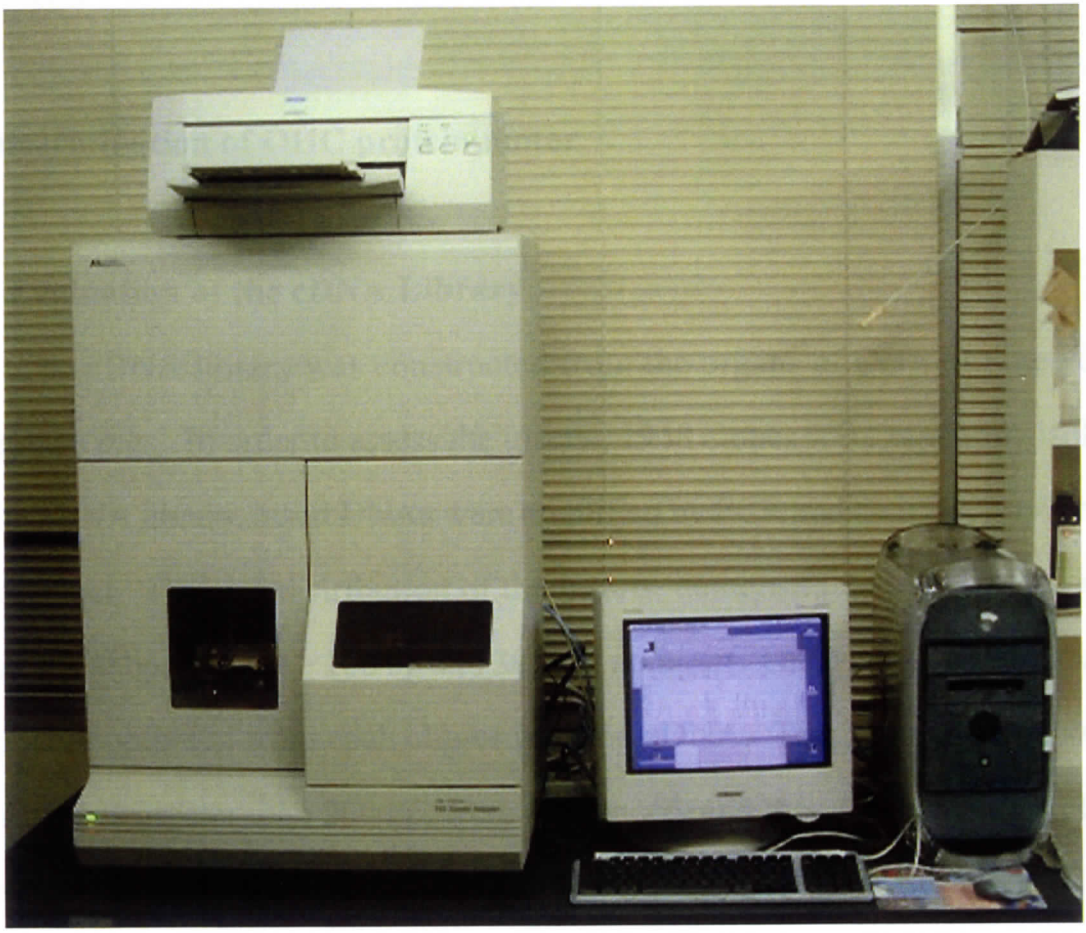


Figure 3.15. The schematic representation of the procedure for combining the adjacent fragments. First, the adjacent fragments were heated to separate these complementary strands. These strands were then annealed with an adjacent strand because these two strands contained the same sequence. As the annealing site served as a primer, DNA polymerase synthesized the DNA downstream from this site. Next, the combined fragment was amplified by PCR. Finally, a large number of combined fragments were obtained.

(a)



(b)

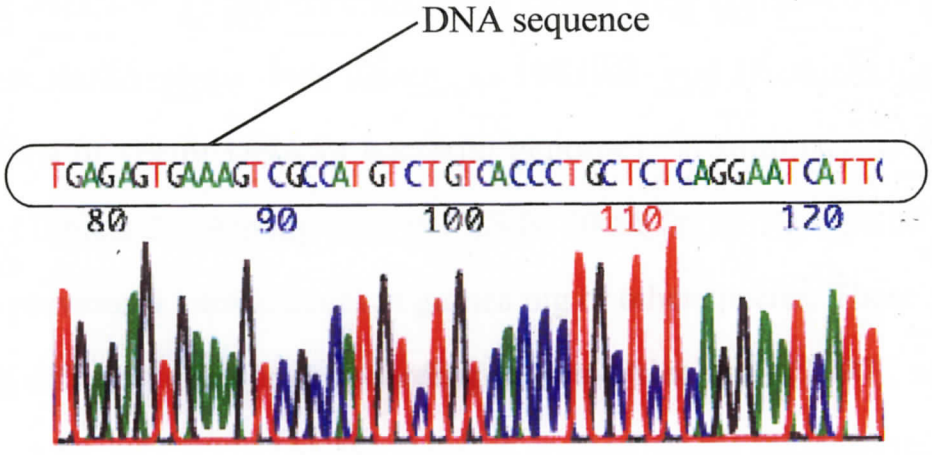


Figure 3.16. (a) Automated DNA sequencer (ABI Prism 310; Applied Biosystems). (b) An example of its output result.

4. Results

4.1. Determination of OHC protein motor

4.1.1. Evaluation of the cDNA Library

The cDNA library was constructed from the organs of Corti of nine young adult guinea pigs. In order to assess the inserts, 1948 clones were randomly isolated from the cDNA library, insert DNAs were amplified by PCR and then electrophoresed in agarose gel. The size distribution of the inserts, demonstrated in Table 4.1, were 45 % at < 200 bp, 17 % at > 200 bp and 38 % at no insert. On average, approximately 150 bp were obtained from each clones inserted cDNA. The 342 clones, of which insert size was greater than 200 bp, were sequenced, and then 127 redundant clones were removed.

4.1.2. Classification of the ESTs

The sequences of non-redundant 235 clones were compared by FASTA 3.0 analysis with DDBJ release 40 to classify as 197 ESTs and 38 contaminants (rRNA and fragments of vector DNA or bacterial genome). A summary of the ESTs is reported in (Table 4.2). Among total 197 ESTs, 70 ESTs (35 %) showed significant matches to previously known genes in guinea pig or other species. These genes were assigned to different categories according to Sato-Prior et al. (1997), as shown in Table 4.3. And, 127 ESTs (65 %) showed no significant matches to sequences deposited in the DDBJ databases, presumed that these ESTs may be novel genes.

Among the 70 clones identified known genes, 5 showed homology with previously identified genes in guinea pig, and the other 65 showed homology with genes in other species, including human, mouse, rat, rabbit, bovine, etc. The most

abundant transcripts observed in our library were the hematopoietic sequences (8.6 %), composed of alpha and beta globins, the mitochondrial genes (7.1 %) and microtubule-associated proteins (4.3%).

4.1.3. Tissue expression analysis of unknown genes

From the 127 clones considered as unknown genes, 39 clones, showing no significant matches to any sequence in the database and optimum sequences for designing PCR primer, were selected for tissue expression analysis using RT-PCR. RT-PCR from 10 different guinea pig tissues (cochlea, cerebellum, kidney, liver, heart, brain, spleen, lung, eye and testis) were performed with each selected clones using specific primers (Table 4.4). As a result of this tissue expression analysis, 26 clones exhibited ubiquitous tissue expression, whereas 13 clones showed a restricted tissue distribution. In particular, clone No.10 was expressed in cochlea, cerebellum and eye, and clone No.12 was exclusively expressed in cochlea, spleen and lung (Figure 4.1).

4.2. Cloning the gerbil prestin cDNA

4.2.1. Cloning prestin four fragments

The four prestin PCR products were electrophoresed on an agarose gel (Figure 4.2). PCR products with length corresponding to the pres1 cDNA fragment (645bp), pres2 (613bp), pres3 (549bp), and pres4 (626bp) were observed at lane 1, lane 2, lane 3, and lane 4, respectively. These four PCR products were subcloned in a plasmid vector for sequencing.

4.2.2. Insert check and sequencing

The transformed *E. coli* colonies were picked up from each agar plate to check the inserts by EcoR I digestion (Figure 4.3). The positive clones were sequenced and these sequences were compared with the prestin sequence by GENETYX-MAC (Table 4.5). The computer analysis revealed that four fragments, pres1-4, derived from the gerbil prestin were obtained.

4.2.3. Combining four fragments

The inserted cDNA which was corresponding to the gerbil prestin sequence was amplified by PCR and the adjacent fragments were combined. The combined fragments of pres1&2 and pres3&4 were electrophoresed on an agarose gel (Figure 4.4(a)). Due to the electrophoresis mobility, the length of pres1&2 and pres3&4 seemed to be matched those of expected region of the gerbil prestin. These combined products were subcloned in a plasmid vector and the insert was checked by EcoR I digestion (Figure 4.4(b)), It is predicted that Pres1&2-1,pres1&2-4, and pres1&2-6 were including the pres1&2 cDNA and Pres3&4-1,pres3&4-2, and pres3&4-5 were including the pres3&4 cDNA. The inserted cDNAs were amplified.

4.2.4. Combining pres1&2 with pres3&4

Pres1&2 was combined with pres3&4, resulting the amplification of ~2.3kbp fragments (Figure 4.5(a)). The PCR product was subcloned in a plasmid vector for sequencing. Eight single-colonies were picked up, followed by the purification of plasmid DNA. The length of inserted DNA was examined by an agarose gel electrophoresis after EcoR I digestion, which demonstrated that four among the eight plasmid-clones possessed ~2.3kbp DNA insert (Figure 4.5(b)). Sequencing analysis revealed that the sequence of two of them were completely matched to that of the

coding region of the gerbil prestin cDNA. However, the other two clones contained a lack of single base in the combined region.

Table 4.1. Summary of isolated clones

Category	Number of clones (%)
Total clones isolated from the cDNA library	1948
Clones (> 200 bp) selected for sequencing analysis	342 (17)
Non-redundant clones	235 (12)
Clones (< 200 bp)	870 (44)
No insert clones	736 (38)

Table 4.2. Summary of the sequenced 235 clones

Category	Number of clones	(%)
Total clones analyzed in databases	235	
ESTs	197	(83)
rRNA	30	(12)
Fragments of vector DNA or bacterial genome	8	(3)
Total ESTs	197	
Known genes	70	(35)
Unknown genes	127	(65)
Clones performed by RT-PCR	39	

Table 4.3. ESTs matched to known genes in DDBJ databases

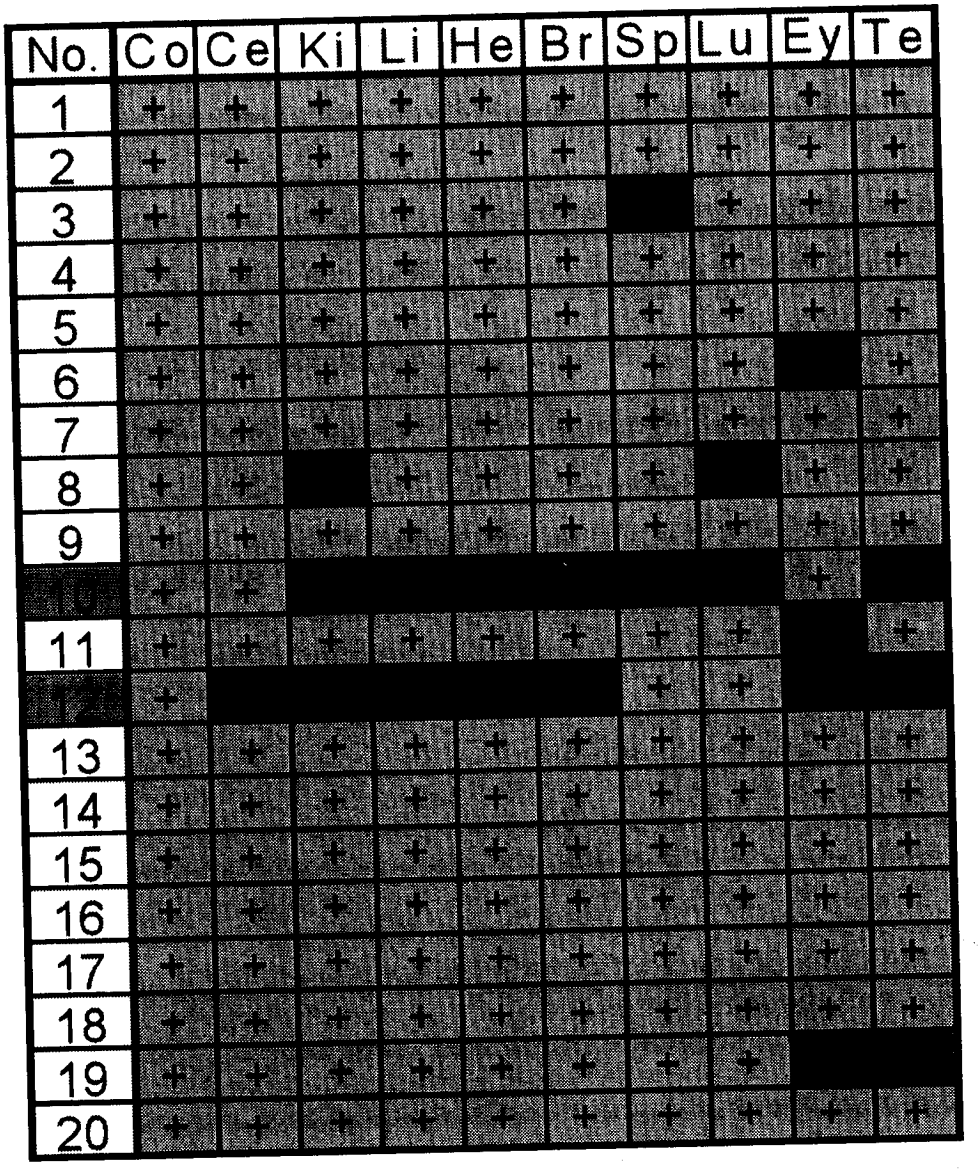
Clones No.	Putative Identified Sequence Name	Species	Accession No.	Score	%	bp overlap/ total bp
Metabolism						
035-08	alpha-lactalbumin	Guinea-pig	Y00726	1047	73	611/705
092-16	alpha-lactalbumin	Guinea-pig	Y00726	607	74	323/330
118-12	BCL2/adenovirus E1B 19kD-interacting protein 2	Human	U15173	652	74	352/341
091-13	beta-cop	Human	AF084457	2826	94	667/667
114-14	co-beta glucosidase	Human	J03077	704	84	197/304
098-16	co-beta glucosidase	Human	J03077	611	76	234/250
085-10	double-stranded RNA-specific adenosine deaminase	Rat	U18942	925	83	259/259
051-06	DRPLA protein	Human	D38529	841	89	230/224
073-10	HEB helix-loop-helix protein	Human	M80627	1185	92	281/281
090-09	IkappaB kinase complex associated protein	Human	AF044195	858	86	230/230
048-04	lithium-sensitive myo-inositol monophosphatase A1	Human	AF042729	1346	91	334/335
082-10	lymphocyte dihydropyrimidine dehydrogenase	Human	U20938	1397	91	318/318
114-04	metalloproteinase inhibitor TIMP-2	Guinea-pig	AF127803	1041	100	229/228
042-03	OS-9 precurosor	Human	U41635	1075	92	251/251
081-06	PAF acetylhydrolase 45 kDa subunit	Bovine	D30615	1905	92	469/461
096-16	PAF acetylhydrolase beta-subunit	Bovine	D49678	199	91	55/339
109-16	pre- splicing factor	Human	AF107405	1068	93	240/319
017-12	prostaglandin D synthase	Bear	D82047	456	84	136/294
033-12	Rab8-interacting protein	Mouse	U50595	877	89	219/220
033-10	retinoblastoma-related protein Rb2/p130	Mouse	U36799	1105	83	304/304
028-12	sphingolipid activator proteins	Human	M81355	840	88	229/229
006-16	ubiquitin-protein ligase E3-alpha	Human	AF067384	325	84	146/279
Structural						
061-14	Collagen Alpha 5(IV)	Human	AL031622	546	75	254/239
021-09	dystrophin	Chicken	X13369	121	76	62/196
091-10	alpha II spectrin	Human	U83867	1053	89	257/257
003-09	ezrin	Mouse	X60671	1510	89	521/521
051-13	I-plastin	Human	L20826	939	92	218/218
015-08	kinesin-2	Human	Y08319	941	96	185/185
081-12	matrin 3	Rat	M63485	2787	96	680/691
053-01	microtubule-associated protein 1a	Human	U38291	696	81	228/228
006-14	microtubule-associated protein 1a	Human	U38291	573	84	157/158
087-08	microtubule-associated protein 1B	Human	L06237	704	94	171/168
063-08	spectrin SH3 domain binding protein 1	Human	U87166	1305	97	295/296
062-09	tight junction protein (ZO-2)	Dog	L27152	639	85	179/180
Cell signaling and transporters						
106-08	chloride channel protein CLC7	Mouse	AF063100	190	75	148/198
052-05	CLCN3	Human	X78520	341	86	154/236
001-14	dynactin	Human	X98801	474	91	106/106
102-15	dynactin subunit (p22)	Human	AF082513	1452	88	408/410
009-06	fructose transporter (GLUT5)	Rat	L05195	161	75	108/129
094-04	KIT protein	Human	U63834	111	77	73/341
102-14	P2X2 receptor splice variant P2X2-1	Guinea-pig	AF053327	1561	100	312/312

Table 4.3. (continued)

Clones No.	Putative Identified Sequence Name	Species	Accession No.	Score	%	bp overlap/ total bp
Transcription factors and translation machinery						
043-13	calnexin (pp90)	C.domesticus	X53616	1609	90	383/383
117-11	elongation factor 1 alpha	O.cuniculus	X62245	1377	91	309/309
013-09	heat shock protein 70A	F.rubripes	Y08576	874	98	175/172
009-09	heat shock protein 90A	C.griseus	L33676	666	85	168/168
013-14	ribosomal protein S21	Human	L04483	1284	89	307/311
063-02	RNA binding protein.	Mouse	X84692	1215	94	278/317
016-07	ubiquitin-like/S30 ribosomal fusion protein	Sus scrofa	U72543	1294	90	350/350
Hematopoietic sequences						
037-02	alpha-globin	Rabbit	J00658	451	91	95/196
014-12	alpha-globin	Rabbit	J00658	423	92	95/247
065-07	alpha-globin	Rabbit	J00658	419	91	95/235
016-16	alpha-globin	Rabbit	J00658	282	88	67/148
022-07	alpha-globin	Rabbit	M11113	267	100	47/106
001-13	beta-like globin	Rabbit	M18818	1181	67	807/813
Mitochondrial genes						
020-09	complete mitochondrial DNA sequence	E.europaeus	X88898	775	78	241/241
016-02	complete mitochondrial DNA sequence	C.simum	Y07726	384	75	148/150
119-07	complete mitochondrial genome	Guinea-pig	AJ222767	1527	100	286/286
061-03	complete mitochondrial genome	Glis glis	AJ001562	935	78	321/323
003-07	mitochondrial translational initiation factor 2	Bovine	L37835	605	81	248/533
Other Sequences						
003-06	(c-myb) gene	Human	U22376	131	80	45/119
092-15	chromosome 16	Human	AC004493	654	93	262/258
009-10	chromosome 17	Human	AC004108	274	78	99/100
035-16	Coch-5B2	Human	AF006740	911	88	222/222
013-15	DNA sequence	Human	AL022577	139	75	60/157
107-06	GAS-7 protein	Rat	AJ131902	961	95	222/222
105-02	integral membrane protein 1	Mouse	L34260	1980	88	515/511
008-04	KIAA0338 gene	Human	AB002336	343	90	81/124
101-15	KIAA0836 protein	Human	AB020643	1278	87	336/337
015-11	multi PDZ domain protein MUPP1	Human	AF093419	490	85	144/206
003-05	trg mRNA	Rat	X68101	1008	75	376/371

Classification of the 70 identified sequences from the guinea pig organ of Corti cDNA library. Scores, percentages of identity and bp overlap/total bp indicate the degrees of the homology to known genes.

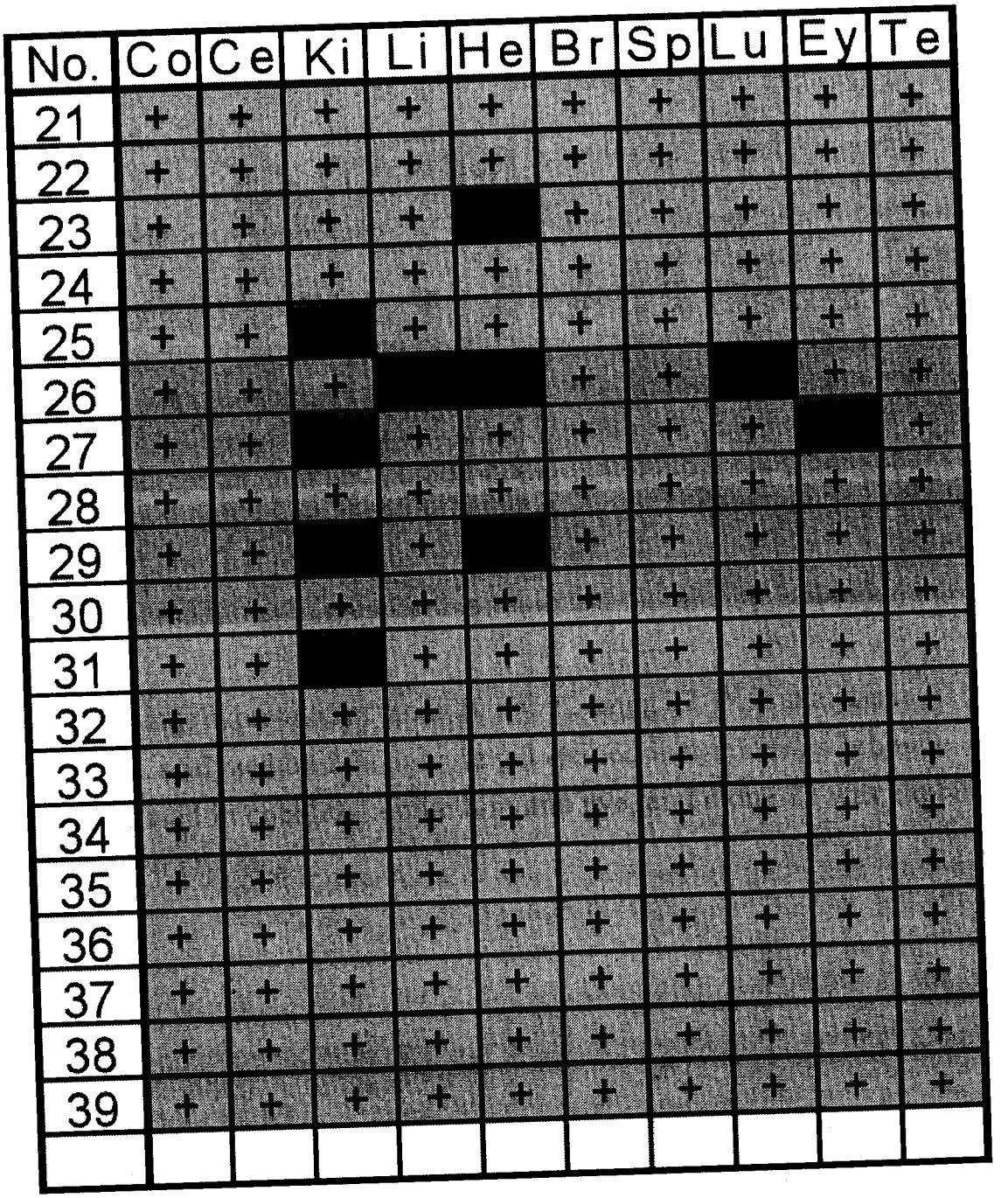
Table 4.4. Tissue expression of unknown clones by RT-PCR



No.	Co	Ce	Ki	Li	He	Br	Sp	Lu	Ey	Te
1	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+		+	+	+
4	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+		+
7	+	+	+	+	+	+	+	+	+	+
8	+	+		+	+	+	+		+	+
9	+	+	+	+	+	+	+	+	+	+
10	+	+							+	
11	+	+	+	+	+	+	+	+		+
12	+						+	+		
13	+	+	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+	+	+
18	+	+	+	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	+		
20	+	+	+	+	+	+	+	+	+	+

The clone number is indicated in the first column. Total 10 tissues (Co, cochlea; Ce, cerebellum; Ki, kidney; Li, liver; He, Heart; Br, brain; Sp, spleen; Lu, lung; Ey, eye; and Te, testis) were tested. A positive result in a given tissue is symbolized by + in the corresponding column.

Table 4.4. (continued)



No.	Co	Ce	Ki	Li	He	Br	Sp	Lu	Ey	Te
21	+	+	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+	+	+
23	+	+	+	+		+	+	+	+	+
24	+	+	+	+	+	+	+	+	+	+
25	+	+		+	+	+	+	+	+	+
26	+	+	+			+	+		+	+
27	+	+		+	+	+	+	+		+
28	+	+	+	+	+	+	+	+	+	+
29	+	+		+		+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+
31	+	+		+	+	+	+	+	+	+
32	+	+	+	+	+	+	+	+	+	+
33	+	+	+	+	+	+	+	+	+	+
34	+	+	+	+	+	+	+	+	+	+
35	+	+	+	+	+	+	+	+	+	+
36	+	+	+	+	+	+	+	+	+	+
37	+	+	+	+	+	+	+	+	+	+
38	+	+	+	+	+	+	+	+	+	+
39	+	+	+	+	+	+	+	+	+	+

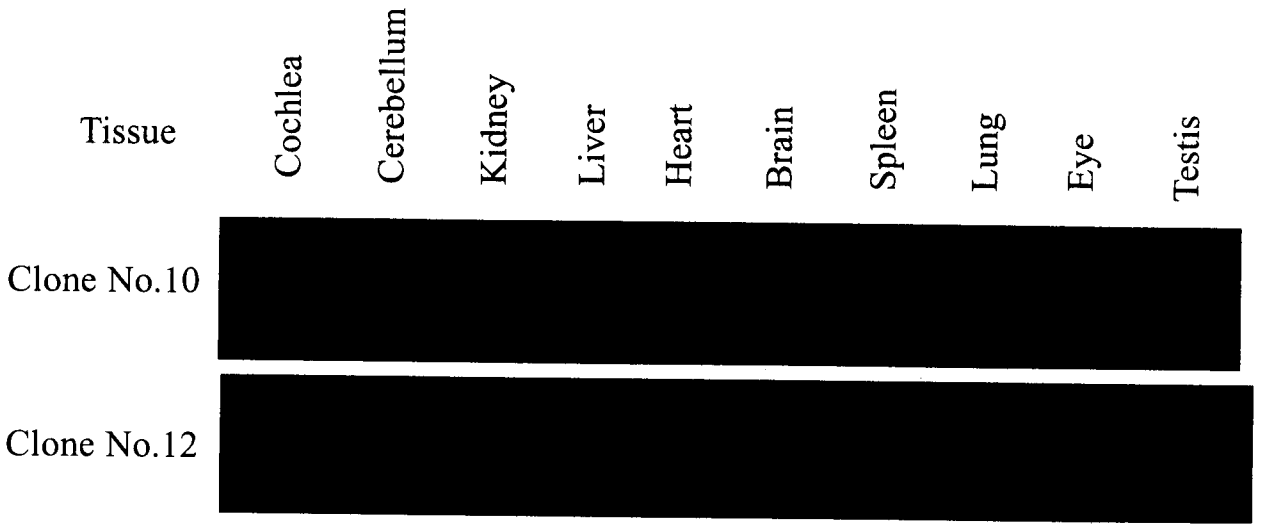


Figure 4.1. Tissue expression of clone 10 and 12 using RT-PCR analysis in 10 tissues. PCR products analyzed on 2 % agarose gel stained with ethidium bromide. Clone 10 was expressed in cochlea, cerebellum and eye, and clone 12 was expressed in cochlea, spleen and lung.

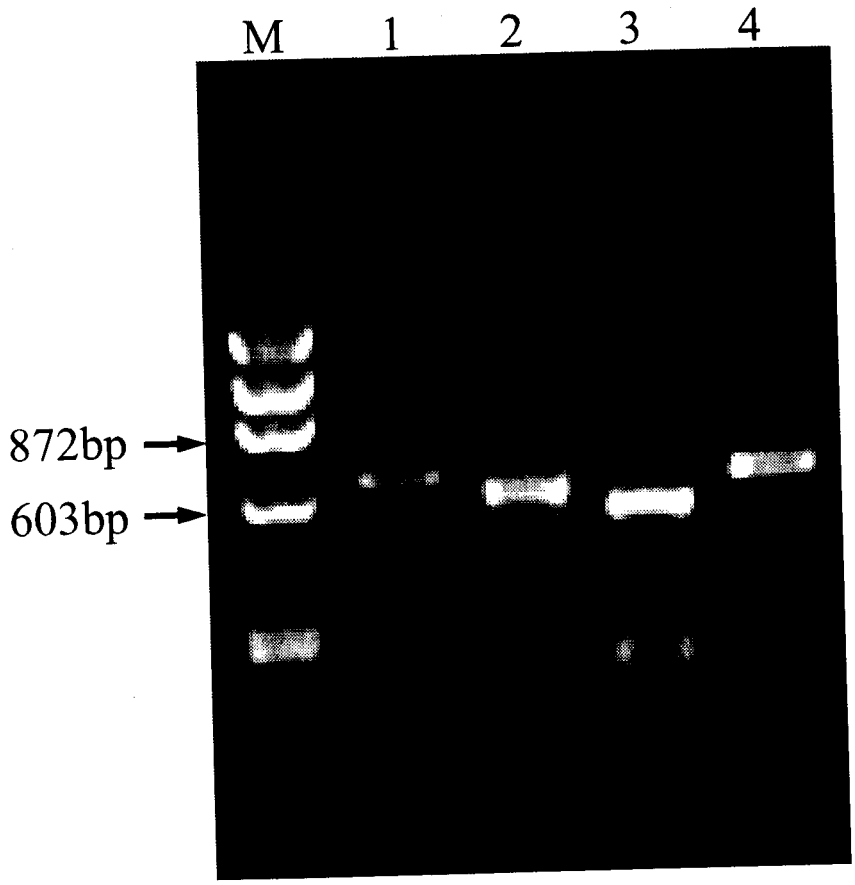
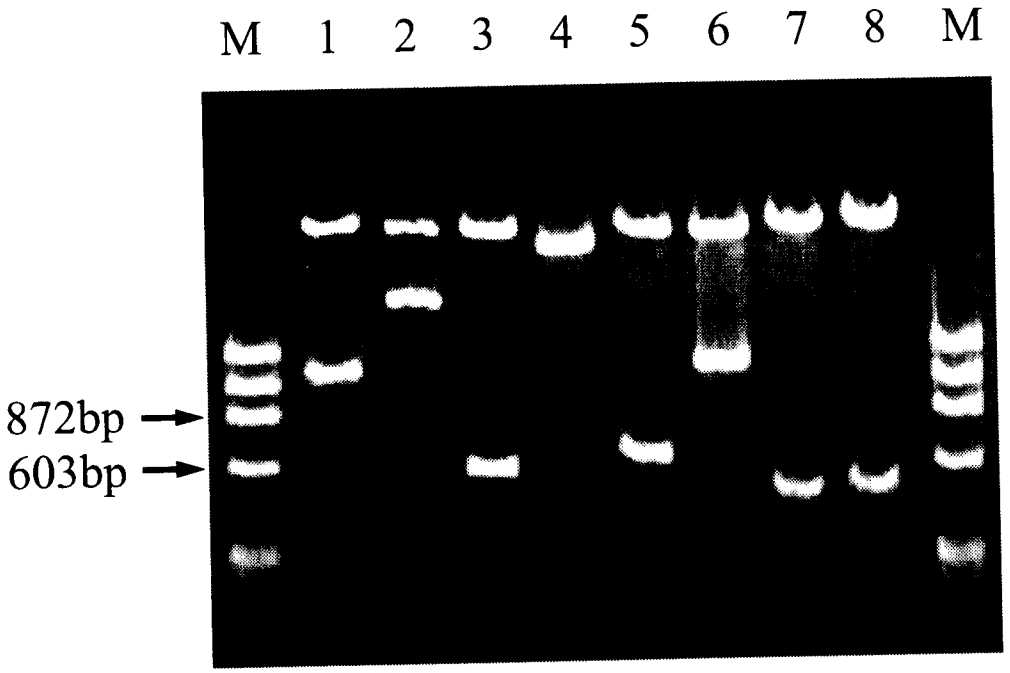


Figure 4.2. Electrophoresis of four pres fragments. Products obtained from mRNA extracted from cochlea were analyzed on 1.5% agarose gel stained with ethidium bromide. The PCR product with length corresponding to the pres1 cDNA fragment (645bp), pres2 (613bp), pres3 (549bp), and pres4 (626bp) were observed at lane 1, lane 2, lane 3, and lane 4, respectively. Lane M contains the ϕX174/*Hae* III molecular size marker.



Lane M : ϕX174/*Hae* III
Lane 1 : pres2-9 (Clone name)
Lane 2 : pres2-10
Lane 3 : pres2-11
Lane 4 : pres2-12
Lane 5 : pres2-13
Lane 6 : pres2-14
Lane 7 : pres2-15
Lane 8 : pres2-16

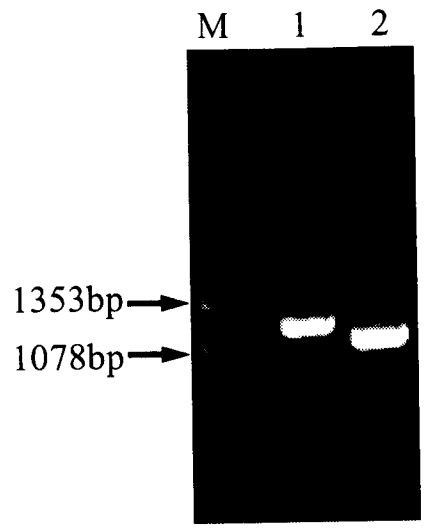
Figure 4.3. Typical electrophoresis patterns of insert check. Plasmids were digested with EcoR I followed by electrophoresis on 1.5% agarose gel. If the insert had been ligated, the estimated DNA size band was observed. In this figure, pres2-11, pres2-13, pres2-15, and pres2-16 were predicted that the pres2 cDNA was ligated. Lane M contains the ϕX174/*Hae* III molecular size marker.

Table 4.5. Summary of isolated clones.

Category	Pres1	Pres2	Pres3	Pres4
Total clones analyzed by restriction enzyme	30	16	6	6
Clones selected for sequence	9	6	5	4
Show matches to prestin sequence	1	1	5	4

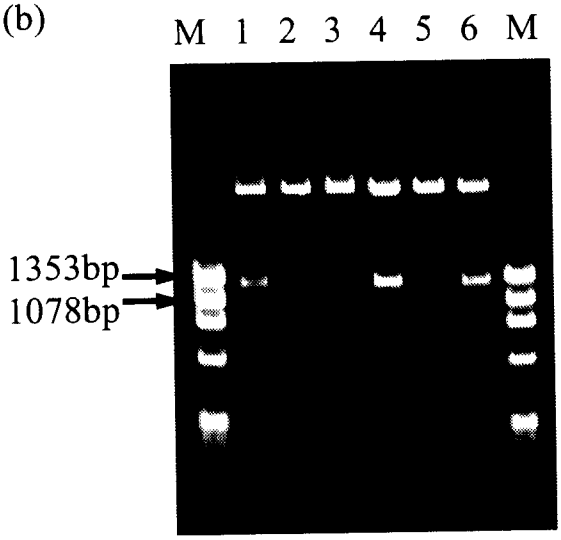
The result of sequences shown in Table 4.1 represents that the subcloning efficiency of pres1 and pres2 was low. The reason might come from the PCR amplification.

(a)

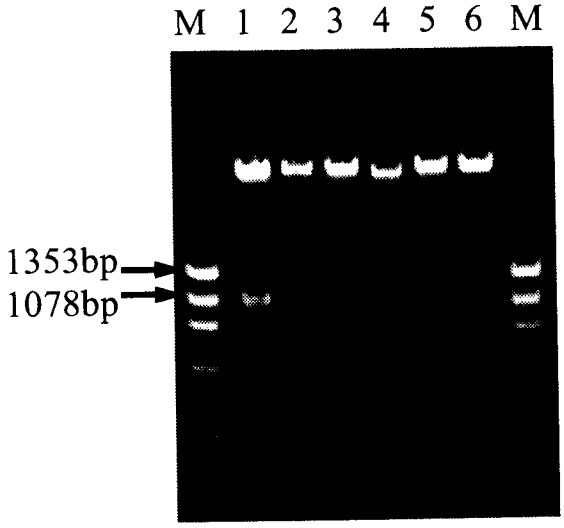


Lane M : ϕX174/*Hae* III
Lane 1 : pres1&2(1236bp)
Lane 2 : pres3&4(1161bp)

(b)



Lane M : ϕX174/*Hae* III
Lane 1 : pres1&2-1 (Clone name)
Lane 2 : pres1&2-2
Lane 3 : pres1&2-3
Lane 4 : pres1&2-4
Lane 5 : pres1&2-5
Lane 6 : pres1&2-6



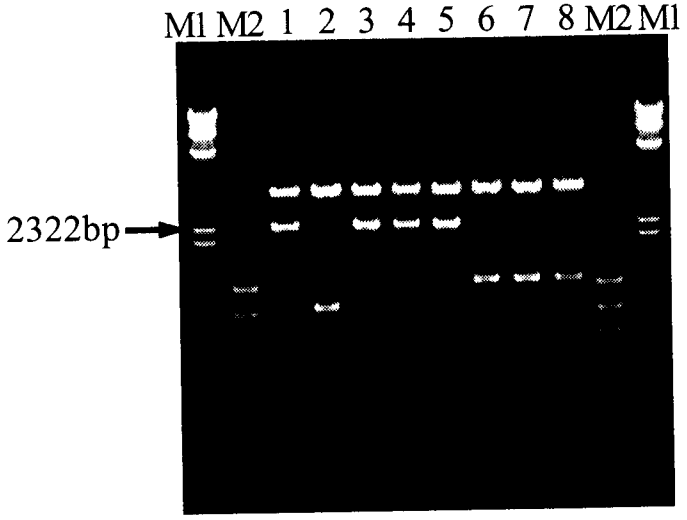
Lane M : ϕX174/*Hae* III
Lane 1 : pres3&4-1 (Clone name)
Lane 2 : pres3&4-2
Lane 3 : pres3&4-3
Lane 4 : pres3&4-4
Lane 5 : pres3&4-5
Lane 6 : pres3&4-6

Figure 4.4. Electrophoresis.
(a) The results of combining pres1 with pres2 (Lane 1) and pres3 with pres4 (Lane2). Due to the electrophoresis mobility, the length of pres1&2 and pres3&4 seemed to be matched those of expected region of the gerbil prestin. Lane M contains the ϕX174/*Hae* III molecular size marker.
(b) The result of insert check. It is predicted that pres1&2-1, pres1&2-4, and pres1&2-6 were including the pres1&2 cDNA and pres3&4-1, pres3&4-2, and pres3&4-5 were including the pres3&4 cDNA. Lane M contains the ϕX174/*Hae* III molecular size marker.

(a)

Lane M : λ/*Hin*d III
Lane 1 : PCR products

(b)



Lane M1 : λ/*Hin*d III
Lane M2 : ϕX174/*Hae* III
Lane 1 : prestin-1 (Clone name)
Lane 2 : prestin-2
Lane 3 : prestin-3
Lane 4 : prestin-4
Lane 5 : prestin-5
Lane 6 : prestin-6
Lane 7 : prestin-7
Lane 8 : prestin-8

Figure 4.5. Electrophoresis picture.
(a) Combining pres1&2 with pres3&4 resulted the amplification of longer fragments, of which the size was approximately 2.3kbp (Lane 1). Lane M contains the λ/*Hin*d III molecular size marker.
(b) The result of insert check. It is predicted that prestin-1, prestin-3, prestin-4, and prestin-5 were including the coding region of the gerbil prestin cDNA. Lane M1 contains the λ/*Hin*d III molecular size marker. Lane M2 contains the ϕX174/*Hae* III molecular size marker.

5. Discussion

5.1. Determination of OHC protein motor

In this study, to identify candidate genes for the OHC protein motor, we constructed a guinea pigs organ of Corti cDNA library. In spite of fewer sacrificed animals than that of previously described cochlear library (Ryan et al., 1993; Robertson et al., 1994; Soto-Prior et al., 1997; Heller et al., 1998), this library contains a comparatively sufficient number of clones to characterize gene expression of guinea pig organ of Corti. Total 197 ESTs were randomly isolated and classified according to their match rate for known sequences deposited in the DDBJ databases.

One of the noticeable features of this library is that unknown genes are fairly abundant (65 % of the total ESTs) compared to percentages described in other libraries such as 37 % in rat cochlea library (Soto-Prior et al., 1997), 22 % in rat OHC library (Harter et al., 1999), 13 % in human cochlear library (Skvorak et al., 1999). The relatively high percentages of unknown genes may be a reflection of the difference of species. The entries of human or rat DNA sequences deposited in the database are one of the tops of all species, however, those of guinea pig are not so many (Human, 2579749 entries; Rat, 82488 entries; Guinea pig, 364 entries. Oct. 1999). This observation is in accordance with that only 5 of 70 known genes showed homology with previously identified genes of guinea pig.

Also among the ESTs are genes that are probably 'contaminants' from the tissue correction, because it was not always possible to entirely remove other tissues bounding the organ of Corti. For example, the hematopoietic tissue transcripts such as alpha and beta globins are more likely to originate from contaminating blood than from tissue of the organ of Corti. However, previous studies performed with antibodies specific to erythrocyte proteins showed a binding of these antibodies to the

OHC lateral plasma membrane, suggesting the presence of protein, mostly cytoskeltal, common to these two types of cells (Knipper et al., 1995; Zine and Shweitzer, 1997).

In spite of those unavoidable situations, the library seems to be highly representative of gene expression in the organ of Corti. For example, Coch-5B2, expressed at very high levels in the cochlea and vestibule, is likely to be a secreted protein. The autosomal dominant human hearing disorder DFNA9 are caused by mutations in the mammalian equivalent of Coch-5B2 (Robertson et al., 1998). Fructose transporter GLUT5, identified immunohistochemically in the basolateral membrane of gerbil OHCs (Nakazawa et al., 1995), is one of candidates for the OHC motor molecule (Geleoc et al., 1999). ATP-gated ion channel, P2X2 receptor, revealed the presence in hair cells of the guinea pig in recent study (Housley et al., 1999).

Even though many of the clones identified largely represent comon genes by RT-PCR analysis, 2 out of 197 clones showed a predominant expression of its mRNA. This would indicate that approximately 1 % clones in this cDNA library represent genes predominantly expressed in the organ of Corti. The percentage in the present study is in accordance with that reported in recent similar cochlear EST studies (Soto-Prior et al., 1997; Harter et al., 1999; Skvorak et al., 1999). It was anticipated that ten or twenty thousand genes are generaly expressed in a cell. This may suggest that a lot of genes responsible for cochlear function remain to be identified.

Clone No. 10 showed expressed in cochlea, cerebellum and eye, and clone No.12 was exclusively expressed in cochlea, spleen and lung. Sequence comparison of No.10 and No.12 clone to those in DDBJ databases show that they are not a previously identified genes. Differential expression of the No.10 and No.12 clone in the organ of Corti, as compared to a wide variety of human tissues ranging from structural to hematopoietic, to other specialized tissues may indicate an important

function for this genes in the organ of Corti.

The cDNA library in this study will provide a valuable reagent to access additional genes that are preferentially expressed in the organ of Corti. Identification and characterization of both novel as well as previously known genes are interest in terms of their role in cochlear function and hearing process.

5.2. Cloning the gerbil prestin cDNA

5.2.1. Subcloning efficiency

The result of sequences shown in Table 4.1 represents that the subcloning efficiency of pres1 and pres2 was low. The reason might come from the PCR amplification. When four prestin fragments were amplified from gerbil cochlear cDNA, the redundant fragments which contain similar sequence to primer were amplified and these fragments were subcloned. Moreover, as the length of these fragments were similar to that of the pres1 and pres2 fragments, these clones were selected for sequence in the insert check. As a result, the subcloning efficiency was reduced.

5.2.2. PCR amplification

It is known that PCR amplification sometimes occurs a misincorpolation. To reduce this effect, KOD DNA polymerase were used in this study. KOD DNA polymerase exhibits a very efficient 3'-5' exonuclease proofreading activity. This characteristic is essential for the correction of misincorpolated nucleotides during PCR. Thus, KOD DNA polymerase shows a high PCR fidelity. However, in this study, the misincorpolation was occurred when amplifying the pres1 fragment and

combining pres1&2 with pes3&4. The pres1 fragment contained one point mutation, which results in amino acid substitution (serine to arginine). This mutation was corrected before the next step by a PCR-based mutagenesis methods. During combining pres1&2 with pres3&4, it was found that a single base was deleted in a half of picked up colonies, resulting in a frame-shift of the prestin cDNA. It means that, although KOD DNA polymerase has proofreading capability, a repetition of PCR yield misincorpolation. Additionally, it is said that the reaction condition of PCR, e.g. annealing temperature or the number of amplification cycles, also caused misincorporation. Thus it is important to consider the reaction condition and total number of PCR.

5.2.3. Further analysis of prestin

In this study, the combining method has enabled us to clone prestin cDNA. For further analysis of prestin, the cloned cDNA will subclone into eukaryotic expression vector and transfect into COS-7 cells. To determine expression of the full-length protein or to investigate functional properties, e.g. voltage-dependent charge movement, in transfected cell will be preformed.

6. Conclusions

The guinea pig organ of Corti cDNA library was constructed. The sequencing analysis revealed that 197 ESTs were possibly derived from the library. The ESTs were then classified as known genes and unknown genes. Moreover, to identify the candidates for the OHC motor molecule, the tissue expression analysis by RT-PCR was performed. The conclusion could be drawn as follows:

1. The cDNA library seemed to be highly representative of gene expression in the organ of Corti.

2. The two putative novel genes presenting a limited expression pattern were found from the cDNA library.

3. A lot of genes responsible for cochlear function remain to be identified.

4. The cDNA library described herein provides a resource for the identification of proteins important for cochlea function.

However, the candidate genes for the OHC protein motor could not be identified. Therefore, next, based on the base sequences of gerbil prestin deposited in the NCBI, an attempt was made to clone the prestin cDNA from gerbil cochlear cDNA by PCR. The results were as follows:

5. The combining method is useful to clone long cDNA by PCR.

6. It is important to consider the reaction condition of combined method.

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