Laboratory Animal Research Center
実験動物研究施設

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Morbilliviruses in the family Paramyxoviridae including canine distemper virus, rinderpest virus and measles virus are highly infectious among their natural hosts. We have succeeded in establishing a system of reverse genetics for these three morbilliviruses, using originally isolated strains. The studies on the functions of viral proteins in replication and species-specificities have been performed. We have also performed the basic research for prevention of Hepatitis C virus infection and hepatocellular carcinoma. In addition, more than 30,000 mice, mainly transgenic and gene-targeted ones, are always kept for researches of IMSUT and the technical staffs contribute to their maintenance and breeding.

1. Development of reverse genetics of morbilliviruses and application to pathological analysis.

Misako Yoneda, Ryuichi Miura, Fusako Ikeda, Akiko Takenaka, Hiroki Sato, Yoshihisa Inoue, Keiji Hirabayashi, Yuri Muto, Akira Watanabe, Masashi Uema, Takahiro Seki, Naoki Kooriyama, Cheiko Imai, Munemitsu Masuda, Tomoko Doi, Tomoki Kubota, Keiko Fukumoto, Yoshinori Fukaya, Kyoko Tsukiyama-Kohara, and Chieko Kai.

The genus morbillivirus, in the *Paramyxoviridae* family, includes 7 viruses; measles virus (MV), rinderpest virus (RPV), canine distemper virus (CDV), peste des petits ruminants virus, and three aquatic mammalian viruses. Morbilliviruses are highly contagious and are considered one of the most important pathogens in each host animal. For a decade from the late 1980s, serious epidemics with mass mortality occurred among both seal and lion populations. These outbreaks were attributed to infection with CDV, although big felids had not previously been considered susceptible to CDV. The mechanisms of pathogenicity and cross-species infection for morbilliviruses are still major problems to be solved. Development of rescue systems of nonsegmented single and negative-strand viruses (*mononegavirales*) since 1994 have opened vast new fields of analysis for a wide range of previously inaccessible areas of these viruses.

We established an excellent animal model for RPV infections using rabbits, which exhibit natural symptoms to experimental peripheral infection. To investigate the pathogenicity of RPV, we isolated ten virus clones from homogenates of infected rabbit lymph nodes by plaque cloning in B95a cells. Among the 10 clones, 2 clones were highly virulent, and one clone was avirulent, defined by histopathology and virus growth in lymphoid tissues. In B95a cells, 8 clones showed almost identical growth curves, while the virus yield of one virulent clone was high and of the avirulent clone was markedly low. Clinical features, virus growth and histopa-

thological changes of rabbits after infection of each virus clones were correlated with the virus growth in B95a cells.

Using the full genome cDNA of the most virulent clone of the RPV-L, we succeeded to develop a reverse genetics system (rRPV-Lv). We first applied a system for the analysis of the role of the V protein produced from the P gene by RNA editing. The V deficient Sendai virus was shown to be avirulent in infected mice and the V deficient Measles virus was shown to induce significantly fewer and milder clinical symptoms with a lower mortality rate than the parental virus in newborn mice. The V deficient rRPV-Lv replicated to the same extent as the parental virus in B95a cells *in vitro*. The rabbits infected with the V deficient rRPV-Lv showed clinical symptoms including fever or immunosuppression and reduced body weight, similar to those infected with the parental strain. In this study, the V protein was shown to exert little effect on virulence in RPV, in contrast with the protein's role in Sendai and Measles virus.

In addition, we previously established a monkey model for MV. This was the first model to demonstrate measles rash with other natural symptoms such as immunosuppression following experimental infection with MV isolated from affected humans. A reverse genetics system has been successfully developed using a field isolate, the HL strain. This system with the animal model could offer another powerful tool for the investigation of the mechanisms of pathogenesis and for the development of polyvalent vaccines for significant human diseases, after the reduction of virulence by genetic engineering.

2. The effect of Rinderpest virus nucleocapsid protein and phosphoprotein on the species specific pathogenicity

Misako Yoneda, Tom. Barrett, Kyoko Tsukiyama-Kohara, and Chieko Kai: [1]Institute of Animal Health, Pirbright, UK

We have previously demonstrated that Rinderpest virus (RPV) haemagglutinin (H) protein plays an important role in determination of host range but other viral proteins are clearly required for full RPV pathogenicity to be manifest in different species from its natural host. To examine the effects of the RPV nucleocapsid (N) protein and phosphoprotein (P protein) on its cross species pathogenicity, we have newly constructed two recombinant viruses. They were rescued from cDNAs of the RPV-RBOK strain that is avirulent in rabbits, in which the H and P or H, N and P genes were replaced with those from the RPV-L strain that is highly pathogenic in rabbits, designated as the rRPV-lapHP or the rRPV-lapNHP, respectively. Rabbits inoculated with the RPV-L became feverish, showed a decrease in body weight gain and leucopenia. Clinical signs of infection were never observed in rabbits inoculated with either the RPV-RBOK or with the rRPV-lapH. However, Rabbits inoculated with either the rRPV-lapHP or with the rRPV-lapNHP became pyrexic and showed leucopenia. Further, high virus titer and histopathological lesions in lymphoid tissues were clearly observed in those of infected with rRPV-lapHP and rRPV-NHP, although those were not observed in rabbits infected with rRPV-lapH. Therefore, RPV P protein is considered to have a key role in the replication of the virus in the cross species infection.

3. A substitution in N protein alters the pathogenicity of rinderpest virus L strain in vivo.

Misako Yoneda, Reiko Sato, Takahiro Seki, Fusako Ikeda, Ryuichi Miura, Kyoko Tsukiyama-Kohara and Chieko Kai.

We analyzed two clones of rinderpest virus (RPV) isolated from the lapinized RPV strain, which is highly virulent in rabbits. One of the clones RPV-Lv retained its virulence, whilst the second, RPV-La was attenuated. Comparison of the gene sequence of the Lv strain and the La strain showed only six nucleotide differences in all genes: G to A at position 1665 in the N gene, A to G at position 2288 in the P and C gene, C to T at position 3099, T to C at position 9694, C to T at position 11117 in the L gene. In the 6 substitutions, only the mutations at $G_{1665}A$ (N), $A_{2288}G$ (P/C) and $C_{11117}T$ (L) direct amino acid changes. To examine which substitutions are implicated in the reduced pathogenicity of the RPV -La strain, we recovered seven infectious RPVs from recombinant cDNAs of RPV-Lv with exchanged N, P and/or L gene from the RPV-La strain by using a reverse genetics system of the RPV-Lv strain. All four recombinant viruses possessing the N gene of the La strain replicated slower than RPV-Lv strain and other recombinants in vitro. They also showed lower pathogenicity in rabbits compared to that caused by the RPV-Lv strain. These results suggest that the substitution in N protein of RPV affects virus pathogenicity in vivo.

4. Expression and characterization of the recombinant swine interleukin-6

Athipoo Nuntaprasert[1], Yasuyuki Mori[2], Ken-

taro Fujita, Misako Yoneda, Ryuichi Miura, Kyoko Tsukiyama-Kohara, Chieko Kai: [1] Facuty of Veterinary Science, Chulalongkorn University, Bangkok; [2]National Institute of Animal Health, Tsukuba

The swine interleukin-6 (SwIL-6) cDNA was cloned by RT-PCR and each expression system of recombinant SwIL-6 in *Escherichia coli*, insect cells, and mammalian cells were developed. Recombinant SwIL-6 produced in bacteria was applied for generation of the polyclonal antibodies. The rSwIL-6 was purified from supernatant of insect cells with a Q-sepharose or anti-SwIL-6 monoclonal antibody (mAb) based immunoaffinity column. The antibodies showed that the molecular weight of rSwIL-6 was approximately 26 kDa in *E. coli*, 25, 26, 30 kDa in insect cells, and 26 and 30 kDa in mammalian cells. These variations of molecular weight were probably due to the different modifications of glycosylation. All these recombinant proteins retained the antigenicity and biological activity on 7TD1 mouse cells.

5. Phylogenetic and restriction fragment length polymorphism analyses of hemagglutinin (H) protein of canine distemper virus isolates from domestic dogs in Japan.

Masashi Uema, Kenjiro Ohashi, Chiaki Wakasa and Chieko Kai

We conducted phylogenetic and restriction fragment length polymorphism (RFLP) analyses of 995 nucleotides of the hemagglutinin (H) gene open reading frame of field isolates of 23 Canine distemper virus (CDV) strains isolated from domestic dogs in Japan between 1982 and 1998. The phylogenetic analysis showed that Japanese field isolates could be separated into three groups. Eighteen out of the 23 strains constituted one cluster consisting of Japanese CDVs, four strains formed a second Japanese CDV group, and only one strain belonged to a group containing foreign CDV strains. By RFLP analysis using *Ssp* I, we could distinguish all the Japanese field isolates from the vaccine strains. Thus, the RFLP method is useful for differentiating the infections with field CDV strains from the vaccine strains in clinical cases.

6. Development of a Measles Virus Vector Targeting Hepatoma cells by expression of single chain antibody against alpha-fetoprotein.

Takahiro Seki, Kyoko Tsukiyama-Kohara, Ying Huang and Chieko Kai

We previously established a reverse genetics system for the measles virus (MV) using a field-isolated MV-HL strain. For the application of this novel MV vector to therapy of Hepatocellular carcinoma (HCC), we constructed recombinant MVs (rMVs) that express a single chain antibody (ScFv) against human alpha-fetoprotein (AFP) by using this system. We constructed two kinds of rMV-αAFPs, which possess ScFv against human AFP fused with the transmembrane domains (TMD) of MV-hemagglutinin (H) (rMV-H-αAFP) or that with vesicular stomatitis virus (VSV)-glyco (G) protein (rMV-G-αAFP). Both ScFv protein were expressed on the cell surface after the infection with these rMVs. The rMV-αAFPs replicated in B95a cells as well as the parent rMV. In contrast, the yield of rMV-G-αAFP in HepG2 cells was significantly larger than that of rMV-H-αAFP or rMV. The rMV-G-αAFP showed greater infectivity in AFP-positive hepatoma cell lines (Hep3B, HepG2, HuH-7, HT-17, Li-7 and PLC/PRF/5) than the parental rMV, and inhibited colony formation in HepG2 cells. These results indicate that expression of ScFv against human AFP fused with VSV-G TMD on rMV induced extensive infectivity in hepatoma cells, and the possibility of using rMV-G-αAFP as an oncolytic agent against HCC.

7. Identification of cell surface antigens expressing in full-length Hepatitis C virus gene persistently expressing cells.

Kyoko Tsukiyama-Kohara, Kohsuke Izumi, Takashi Takano, Kentaro Saito, Miyuki Awakuni, Ying Huang, and Chieko Kai

Hepatitis C virus (HCV) frequently causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). Since the effective infection system of HCV has not been established *in vitro* and *in vivo*, we previously established the conditional full-length HCV gene expressing HepG2 cells. The HCV expressed and passaged cells increased their tumorigenicity after 48 days passage. In order to clarify the existence of cell surface antigens whose expression level has been changed according to the tumorigenicity, we produced monoclonal antibodies (mAbs) by immunizing the passaged cells. Over one thousand monoclonal antibodies were established, and their reactivities to HCV gene expressing cell lines were characterized. Several mAb clones with significantly different reactivities to HCV gene expressed and passaged cells were obtained. Among them, the mAbs 2-152, 243, and 433 recognized 55, 70 and 30 killodalton (kDa.) molecules, respectively. All these molecules were expressed on the surface of hepa-

toma cell line higher than that of normal hepatic cell line. Expression levels of these molecules were also examined in cancerous and non-cancerous region of tissues from hepatocellular carcinoma in patients' liver with or without HCV and Hepatitis B virus (HBV) infection. Upregulation of the p55 molecule expression in cancerous regions in liver of all HCV infected patients was observed. Thus, p55 molecule might be implicated in occurrence of HCC in HCV positive patients.

In vivo visualyzing system of human hepatoma cells is now trying to be established, using luciferase gene transfeced human hepatoma cells and in vivo imaging system (Xenogen), in order to valid the tumor targeting therapy.

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Amami Laboratory of Injurious Animals
奄美病害動物研究施設

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The Amami Laboratory of Injurious Animals was established in 1965 at Setouchi-cho in Amami-oshima Island in order to study on endemic diseases involving parasite, arthropods, and venomous snakes in the tropics or subtropics.

The Amami-oshima Island belongs to the Nansei (Southwest) Islands and the fauna is quite different from that in other islands of Japan. Since establishment of the laboratory, trials have been carried out to utilize small mammals found unique in the Amami islands as experimental animals in addition to studies on prevention of Habu bites. As well known, successful eradication of filariasis from this island is one of the monumental works of the laboratory. Our present works are as follows:

1. Research on the Habu control

Shosaku Hattori, Yuko Katakai, Hiroshi KIhara[1], Motonori Ohno, Naoko Ueda[2], Shigenari Terada[3], Hiro Yonezawa[4], Yoshihiro Hayashi[5], Michihisa Toriba[6], Hiroyuki Takahashi[7], and Tomohisa Ogawa[8],: [1]Bioscience research Institute, Takara Shuzo Co., Ltd., [2]Departmen of Applied Microbiology, Kumamoto Institute of Technology, [3]Department of Biochemistry, Faculty of Science, Fukuoka University, [4]Department of biochemistry, Faculty of Science, Kagoshima University, [5]Department of Veterinary Anatomy, Faculty of Agriculture, University of Tokyo, [6]The Japan Snake Institute, [7]Department of Genetics, [8]Faculty of Agriculture, Tohoku university

Snake bites by the venomous snake Habu, *Trimeresurus flavoviridis*, have been reported annually about 80 cases in the population of 100,000 in the Amami Islands. Moreover, there is no indication that the population of the Habu itself has decreased, despite a campaign for capture of snakes by the Kagoshima Prefectural Government. Rat-baited box traps have been introduced to catch the snakes and found to be quite effective. However, maintenance of live rats requires man power and its cost is expensive. Therefore, our effort has been focused on the development of attractant for Habu. The attractant extracted from rats seems ineffective if compared with use of live rats.

It was known that the Habu survived the injection of the Habu venom since early times, because some proteins in the serum of the Habu blood combine to the elements of the Habu venom. The research of these binding proteins has been initiated with an objective of clinical trials. Phospholipase A2 and its isozymes isolated from Habu venom have myonecrotic activity and hemorrhagic activity, and metal protease has hemorahagic activity. The binding proteins isolated from serum of Habu inhibit myone-

crotic activity of phospholipase A2 and its isozymes. We found that protein-HSF and peptide-AHP isolated from the Habu serum effectively control the hemorrhage caused by venom of the Habu, *Ovophis okinavensis*, *Agkistrodon blomhoffi brevicaudus, Calloselasma rhodostoma, Bitis arietans, Bothrops asper*, and, *Trimeresurus stejnegeri*.

Further, a statistics analysis and the simulation were done with the snakes captured by the Government, and the analysis of population dynamics of Habu was attempted. As a result of investigating the individual measurement data of the captured Habu over 9 years, we were able to obtain the generous age composition of the Habu. From analyzing of the age pyramid of the Habu and the result of questionnaire surveys for the inhabitant in the Amami-oshima Island, the total population of the Habu which lives in this island was estimated at about 80,000. By the analysis of the measured data of last nine years, the snake sizes were miniaturized, and the population of young snakes decreased. According to these investigations, the population of the Habu is expected to decrease in the near future.

These studies are supported by grants from the Ministry of Land, Infrastructure and Transport and the Kagoshima Prefectural Government.

2. Research on endocrine of the Habu

Takeshi Kuraishi, Misako Yoneda[10], Shosaku Hattori, and Chieko Kai[10]: [10]Laboratory Animal Research center

We determined the cDNA sequence encoding the prolactin (PRL) gene of Habu. The full-length cDNA of Habu PRL consists of 989 bp. The open reading frame encodes a protein of 229 amino acids, which consists of a putative signal peptide of 30 residues and a mature protein of 199 amino acids. The amino acid sequence of Habu PRL deduced from its nucleotide sequence showed higher homologies with those PRL sequences of tetrapod animals than with those of teleosts. Northern blot analysis revealed the Habu PRL mRNA size to be approximately 1.1 kb. The expression of PRL gene in different tissues (brain, pituitary, venom, heart, lung, liver, pancreas, spleen, kidney, testis, ovary, muscle) was determined using northern blot analysis. The high levels of PRL gene expression was only detected in pituitary. PRL is known to have a wide spectrum of functions. In submammalian vertebrates, PRL acts as an osmo -regulatory hormone. The initial step in the action of PRL, like all other hormones, is the binding to a specific membrane receptor, the PRL receptor (PRL-R). In reptiles, presence of PRL-R from kidney membranes of the turtle and snake were reported. The presence of PRL-R in the snake kidney suggests a putative osmoregulatory role for PRL in this reptile. It is thought that Habu PRL is also involved in water and electrolyte balance.

3. Reproduction of squirrel monkeys.

Shosaku Hattori, Takeshi Kuraishi, Yuko Katakai[11], Mamoru Ito[12], and Chieko Kai[10]: [11] Corporation for production and research of laboratory primates, [12]Laboratory of Immunology, Central Institute of Experimental Animals

The squirrel monkey, *Saimiri sciurea*, is widely distributed in the tropical rainforest in Central and South America between 10 degrees N and 17 degrees S of latitudes. The advantage of using this species for medical researches resides in its small size and gentle behavior. In this laboratory, about 5 newborns are given annually by 25 adult females.

The aim is to optimize the use of the non-human primate model in future the Amami Laboratory research activities. The laboratory newly established experimental infection systems which require or can be adapted to the squirrel monkey model, particularly the study of human falciparum malaria. Development of parasites, immune response to malaria parasites and pathological changes were investigated in in-vivo condition, further more, in vitro analysis of cell and molecular level was performed. It is also investigating the mechanisms of infection in immunology, vector development, a vaccine production program, and a clinical trials program.

4. Research of wild mammals.

Shosaku Hattori, Yoshihisa Noboru, Takeshi Kuraishi, Hideki Endo[13], Kimiyuki Tsuchiya[14], Nobuo Ishii[15] and Fumio Yamada[16]: [13]Department of Zoology, National Science Museum, [14] Experimental Animal Center, Miyazaki Medical College, [15]Japan Wildlife Research Center, [16]Wildlife Ecology Laboratory, Forestry & Forest Products Research Institute

Amami-oshima Island is a habitat of animals and plants indigenous to the Nansei Islands. These animals occur originally in the Oriental region of Asia and include the Amami rabbit, *Pentalagus furnessi*, the Ryukyu spiny rat, *Tokudaia osimensis*, the Okinawa long-haired rat, *Diplothrix legata*, the Watase's shrew, *Crocidura*

watasei, and the Musk shrew, *Suncus murinus*. These mammals are used for researches on comparative anatomy, taxonomy, and development of experimental animals. Besides, these mammals are valuable species biologically as survivors from the Miocene about 10,000,000 years ago. We have initiated the investigation for these species to protect from extinction. We have documented the feasibility of recovering large numbers of oocytes from the Watase's shrew, and some of oocytes can be induced to mature in vitro.

Recently, the Java mongoose, *Herpetologica javanicus* grew in the wild as invasive carnivore in the Amami-oshima Island. The population of the mongoose increases every year and the habitat range is extending to south area in the Island. It is necessary to remove the invader to defend nature. Then we are investigating the influence which the mongoose gives to wildlife in the Island. Since hairs such as Amami rabbit, Ryukyu spiny rat, Akahige were confirmed from the excrement of the mongoose, the necessity of the urgent ridding countermeasure of the mongoose was indicated. From 2000, the capture project of the mongoose was started by Ministry of Environment in order to protect Amami-oshima's endemic species.

In this laboratory, a sex determination method using DNA extracted from feces has been developed for Amami rabbit in order to apply fecal DNA analysis to field study of this rabbit. We determined a partial sequence of the ZFX and SRY gene of Amami rabbit. The ZFX gene exists on the X chromosome in both females and males, while the SRY gene exists only on the Y chromosome in males. Based on a partial sequence of the ZFX or SRY gene of Amami rabbit, we designed primers which could amplify DNA fragments of the ZFX or SRY gene. PCR products were detected in 5 out of 7 fecal samples collected from carcass of Amami rabbit and the sexes estimated by the present method were perfectly matched with the actual sexes. Further research is needed to verify from Amami rabbit feces collected in the field. By applying this method in combination with individual identification using fecal DNA analysis to the ecological study of Amami rabbit, it is hoped that we can obtain more information (genetic structure of population, home range, population density) which is necessary for planning appropriate wildlife management in Amami-oshima Island.

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Laboratory of Molecular Genetics
遺伝子解析施設

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This laboratory has two main activities, development of efficient expression vectors for gene therapy and supporting the researchers by advising on recombinant DNA technology under the safety guideline.

The purpose of our laboratory are concerned about not only research but also support for all researchers in this institute. Our supporting activity is involved in advising service on gene-manipulation experiments under the safety guideline. For the research part, we intend to develop novel methods or new experimental systems leading in the field of gene expression and its regulation. We are concentrating mainly on developing efficient adenovirus expression vectors aiming at gene therapy. We are maintaining more than 20 collaborations within and outside of this institute. In these collaborations, we offer and supply our efficient method (COS-TPC method, Miyake *et al*., Proc. Natl. Acad. Sci. USA 93:1320-1324, 1996) to construct recombinant adenovirus (rAd) expressing various genes efficiently. And recently we developed the new cosmid cassette for construction of rAd which can choose not only COS-TPC method but also an easier method using a full-length viral genome with intact viral termini. This new cassette is on sale by Takara Bio INC. and Nippon Gene INC. We have also developed a method for ON/OFF switching of gene expression in mammalian cells using a combination of adenovirus vector and Cre/*loxP* system (Kanegae *et al*., Nucleic Acids Res. 23:3816-3821, 1995; Kanegae *et al*., Gene 181:207-212, 1996) as well as FLP/FRT system (Nakano *et al*., Nucleic Acids Res. 29:e40, 2001). The method will promote many fields of molecular biology and medicine and may open a new field of "intracellular gene manipulation". The research activities in 2004 were shown below.

1. Development of an efficient method for constructing first-generation adenovirus vectors using double-reciprocal recombination mediated by FLP recombinase.

Saki Kondo, Masakazu Nakano, Yuzuka Takahashi, Yumi Kanegae and Izumu Saito

We are developing that a new method for constructing adenovirus vector using double-reciprocal recombination mediated by the site-specific recombinases, typically Cre derived from P1 phage and FLP derived from *Saccharomyces cerevisiae*. A pair of DNA sequences flanking with the wild-type (wt) target sequence (*loxP* for Cre and FRT for ELP) and a mutant target sequence, which recombines efficiently with the identical mutant sequence but not with the wt target, can efficiently be exchanged through double-reciprocal recombination medi-

ated by the site-specific recombinase. We prepared a "recipient virus" which contains an replacing gene flanked with wt and mutant target sequence and also the viral packaging signal flanked with a pair of wt sequences and a "donor plasmid" which contains the viral packaging signal and a purpose gene to be transferred onto the adenovirus genome replicating in recombinase-expressing 293 cells. Until last year, we have established such methods using Cre/*loxP* system and the mutant *loxP* V, which contains two certain transversion mutations in its 34-nucleotide sequences. Since the *loxP* V was identified in our laboratory (Lee and Saito, Gene 216:55-65, 1998), it has been utilized in many researches (cited in Kondo *et al*., Nucleic Acids Res. 31:e76, 2003) and its efficiency and strictness have already proved. We demonstrated that the construction of the first-generation adenovirus vector (E1-substituted type) using this new method was so efficient that the resulting purpose gene-containing rAds reached to >97% of the virus stock only after three cycles of serial passages through the Cre-expressing 293 cell line. However, when this method using Cre was applied to the construction of the helper-dependent adenovirus vector ("gutted vector"), the purpose virus was indeed generated but the titer obtained was rather low. Then another site-specific recombinase FLP was applied to the construction of the E1-substituted rAd, because the mutant FRT which was identified by us (Nakano *et al*., Microbiol. Immunol. 45:657-665, 2001) had not used to this method yet. Because the efficiency of recombination by FLP was lower than Cre, we adopted two approaches to overcome this problem. One was construction of the humanized FLPe (hFLPe), which contains substituted four amino acid sequences derived from FLPe, enhancing thermostability at 37℃ compared with wt FLP, and 19% of silent mutations to "humanize" FLPe codon usage. The other was generation of 293 cell lines expressing FLP efficiently, because the Cre-expressing 293 cell line showed 70-80% of the recombination efficiency at best probably due to the toxicity of Cre. We selected the FLP- and hFLPe-expressing 293 cell lines and tried the above method instead of using Cre. We obtained purpose rAd clones at almost 100% efficiency even in the second viral stock. The results showed that, although recombination efficiency of FLP is generally lower than that of Cre, the efficiency of recombination by FLP- and hFLPe-expressing 293 cells was higher than Cre.

2. Generation of helper-dependent adenovirus vectors using 293 cell lines expressing site-specific recombinase FLP

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Although we have established the method to generate first-generation adenovirus vectors using the double reciprocal recombination of Cre and FLP, generation of gutted vector by this method using Cre needed to improve its efficiency. In the approach of the construction of first-generation rAd described above, it was proved that the 293 cell lines expressing FLP or hFLPe gave higher efficiency of vector generation than those expressing Cre. To examine this reason, we compared the recombination efficiency among 293 cell lines expressing FLP (F 13), hFLPe (H24) and Cre (N11). The results showed that the FLP- and hFLPe- expressing cell lines showed very high excision efficiency of almost 100% when using the analysis of the recipient rAds above. It is important to excise the packaging signal (Ψ) of recipient virus efficiently by site-specific recombinase especially for enrichment of the gutted vector. Because the gutted vector requires helper virus, which supplies all the viral gene products *in trans*, the method for construction and production of gutted vector needs continuous addition of the recipient virus as helper virus at each step of serial passages. So the FLP- and hFLPe-expressing cell lines seemed to be useful for gutted vector construction and enrichment. We constructed "recipient virus" and "donor cosmid" for this purpose and infected/transfected them to FLP- or hFLPe-expressing 293 cells. After only three passages, we detected desired gutted vector, while it took more than seven passages using Cre-expressing cells. And we were able to enrich the gutted vectors using FLP- and hFLPe-expressing cells. So these cell lines expressing FLP appeared to be useful in constructing the gutted vectors.

3. Mutant FLPs aiming for efficient recombination in mammalian cells

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Although the site-specific recombinase FLP can be applied for gene regulation in mammalian cells and for generation of rAd through the double reciprocal recombination, the recombination efficiency of FLP is much lower than that of Cre even at 30℃, which is optimum tempera-

ture of FLP. If FLP variants showing higher activity could be obtained, the application of FLP would become much more popular. We compared the recombination activity of hFLPe with wt FLP by adenovirus expression vector in mammalian cells. The hFLPe-expressing rAd, AxCAhFLPe, gave much higher level of expressed hFLPe and FLP proteins in mammalian cells than the wt FLP-expressing rAd, AxCAFLP. Unexpectedly, however, the recombination efficiency using AxCAhFLPe was no higher than that using AxCAFLP. In addition, we observed cell toxicity when using AxCAhFLPe, that was never observed using AxCAFLP even at a very high dose. To examine the cell toxicity caused by hFLPe, we newly constructed "hFLP" gene, of which the codon usage were "humanized" but the amino-acid sequences are identical to wt FLP, and compared the recombination activities among wt FLP, hFLPe, and FLPe by transfecting each FLP-expressing plasmid to mammalian cells. The western blot analysis showed that hFLP-expressing plasmid gave higher level of FLP protein than FLP-expressing plasmid in mammalian cells. However, the recombination efficiency of hFLP was again no higher than that of FLP. The results suggested that the cell toxicity may not be due to alternation of protein structure by the four amino-acid substitution that stabilize FLP. Now we have newly constructed hFLP- and FLPe-expressing rAds to examine further the recombination efficiency and cell toxicity of these FLP variants in mammalian cells.

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