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1 **Gene Expression of *Autographa californica* multiple**
2 **Nucleopolyhedrovirus (*AcMNPV*) in Mammalian Cells and**
3 **Upregulation of Host β -actin Gene**

4

5 Ryosuke Fujita¹, Takahiro Matsuyama^{1, 2}, Junya Yamagishi^{1, 3}, Ken Sahara¹, Shinichiro
6 Asano¹, and Hisanori Bando^{1*}

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8 Laboratory of Applied Molecular Entomology, Division of Applied Bioscience,
9 Graduate School of Agriculture, Hokkaido University, Sapporo 001-8589, Japan¹
10 Research & Development Center, Santen Pharmaceutical Co., Ltd.
11 Takayama-cho Ikoma-shi, Nara, 630-0101, Japan²
12 Boyce Thompson Institute, Cornell University
13 Tower Road, Ithaca, NY 14853-1801, USA³

14

15

16 Corresponding author: Dr. Hisanori Bando

17 Laboratory of Applied Molecular Entomology, Division of Applied Bioscience,
18 Graduate School of Agriculture, Hokkaido University, Sapporo 001-8589, Japan Tel.
19 011-706-2487 Fax. 011-716-0879
20 e-mail. hban@abs.agr.hokudai.ac.jp (to Hisanori Bando; correspondent)

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1 **Abstract**

2 The gene expression of *Autographa californica* multiple nucleopolyhedrovirus (*AcMNPV*)
3 was examined in two types of mammalian cells, human HeLa14 and hamster BHK cells.
4 DNA microarray analysis followed by RT-PCR identified at least 12 viral genes transcribed
5 in both HeLa14 cells and BHK cells inoculated with *AcMNPV*. 5' RACE was carried out to
6 examine the transcriptional fidelity of these genes in HeLa14 cells. The transcription of *ie-1*,
7 *ie-0* and *gp64* was initiated at a baculovirus early gene motif, CAGT, accompanied by a
8 TATA motif. In addition, the same splicing observed for *ie-0* mRNA in Sf9 cells occurred in
9 HeLa14 cells. While the transcription initiation sites for *pe38* and *p6.9* were not located in
10 the CAGT motif, most of them were in a typical eukaryotic RNA polymerase II promoter
11 structure (a conventional TATA motif and/or an INR). Interestingly, the expression of
12 *β -actin* was upregulated in the mammalian cells inoculated with *AcMNPV*. Subsequent
13 experiments using UV-inactivated virus confirmed the upregulation, suggesting that *de*
14 *novo* synthesis of viral products is not required for the event. These results indicated that
15 the *AcMNPV* genome acts as a template for transcription in mammalian cells through the
16 usual infection pathway, though there is no evidence for the functional expression of viral
17 genes at present.

18

1 **Introduction**

2 *Autographa californica* multiple nucleopolyhedrovirus (*AcMNPV*), a type member of the
3 insect-specific virus family *Baculoviridae* (genus *Nucleopolyhedrovirus*, NPV), infects
4 several important pest insects and has been used as a safer biopesticide in Integrated Pest
5 Management (IPM) program. The nucleopolyhedroviruses are also used as a gene-transfer
6 vector in insect cells, providing one of the most efficient systems for the expression of
7 foreign genes in eukaryotic cells (19). Furthermore, recent studies demonstrated that
8 *AcMNPV* can enter the nucleus of mammalian cells independently of the cell cycle without
9 multiplication (28, 29) and effectively express foreign genes (15) without expressing its
10 own genes (28), suggesting that the baculovirus can be used as a low risk viral vector in the
11 field of gene therapy (10). Recently, *AcMNPV* was shown to be capable of stimulating
12 production of interferon (IFN) in mammalian cell lines and when injected into mice
13 conferred protection from lethal infections of encephalomyocarditis virus (13). It was also
14 reported that the intranasal inoculation of a wild-type *AcMNPV* induced a strong innate
15 immune response, which protected mice from a lethal challenge of influenza virus, through
16 the Toll-like receptor 9 signaling pathway (1, 3). These reports indicate that baculoviruses
17 affects the physiology of mammalian cells though they are not pathogenic to and have no
18 ability to replicate in mammalian cells.

1 NPV is a large rod-shape virus with a double-stranded closed circular DNA genome
2 (130~160 kbp) containing approximately 150 genes (4). NPV genes are usually categorized
3 into 4 groups; immediate-early, delayed-early, late, and very-late (8). After infection, the
4 immediate-early and delayed-early genes are transcribed by host RNA polymerase II and
5 their promoters are characterized by the transcription initiation site consensus motif CAGT
6 (25). Most of them are thought to code for the transcriptional transactivator of viral genes
7 (33). Late and very-late genes are transcribed by viral RNA polymerase from the late
8 promoter motif TAAG (22, 24) whose expression is regulated by the early genes. Thus, the
9 senseful expression of early genes is a key to NPV infection. The mechanism of
10 transcription by RNA polymerase II is highly conserved among eukaryotes (14). Indeed,
11 the promoter for immediate-early gene 1 (*ie-1*) of *Bombyx mori* NPV (*BmNPV*) showed the
12 ability to promote transcription in mammalian cells in transfection experiments using
13 reporter plasmids (21). Furthermore, the viral essential regulatory protein encoded by *ie-1*
14 (*IE-1*) is functional in mammalian cells (21, 23). More recently, the acidic activation
15 domains (AADs) of *AcMNPV* *IE-1* were demonstrated to be functional for transcriptional
16 activation in mammalian cells (12). These observations suggest that NPV could express at
17 least part of the viral genomic information in mammalian cells and may affect the
18 physiology of the host cells. Progress in the application of baculoviruses requires more

1 information about the functions of *AcMNPV* in mammalian cells. In this study, we
2 reexamined the gene expression of *AcMNPV* in mammalian cells using a DNA microarray,
3 which suggested transcription from several viral genes and increased transcription from
4 cellular *β-actin*.

5

6

7 **Materials and Methods**

8 **Cells and viral inoculation**

9 *Spodoptera frugiperda* (Sf) 9 cells were grown in TC-100 medium (Sigma) containing 10%
10 FBS at 26°C. Mammalian cells (HeLa14 and BHK) were maintained in MEM (Nissui)
11 containing 10% FBS in a CO₂ incubator. Treatment (inoculation) of the mammalian cells
12 with *AcMNPV* was carried out by incubating the cells in DMEM (FBS-free) containing
13 *AcMNPV* for 2 hrs at 37°C followed by culture in DMEM containing 10% FBS at 37°C.

14

15 **Viruses**

16 *AcMNPV* was grown in Sf9 cells in TC-100 medium containing 10 % FBS at 26°C. We
17 constructed the recombinant *AcMNPV*, Ac-dhsp-EGFP, using the Bac-to-Bac system
18 (Invitrogen). This virus has an EGFP-coding sequence under the control of the *Drosophila*

1 heat shock protein 70 promoter (nucleotides (nts) 1 to 1927, GB No., AY032740). Viral
2 titers were determined in Sf9 cells by plaque assay according to Maeda's method (19).
3 Inactivation of viruses was carried out by exposing the viral suspension (3×10^7 pfu/ml
4 DMEM) in a sterile tissue culture dish to shortwave UV (UVC, 254nm) at a distance of 30
5 cm for 30 min in a laminar flow hood. The inactivation of infectivity was verified by a
6 plaque assay with Sf9 cells. The recombinant virus carrying *luciferase* under the control of
7 the *ie-1* promoter (rBACie1-luc) (2) was also used to examine the inactivation of viruses
8 and demonstrated that UV-irradiation for 30min under such conditions resulted in the loss
9 of 99.7 % of the activity to express *luciferase* and a lack of plaque formation (data not
10 shown). The approach (irradiation for 30 min) was then used to prepare UV-inactivated
11 virus.

12

13 **DNA microarray**

14 A microarray analysis using a baculovirus DNA chip consisted of 192 spots including 140
15 *AcMNPV* genes was carried out as described elsewhere (32). In brief, total RNA was
16 isolated using Trizol reagent (Invitrogen) and 400 pg of synthesized RNA, λ polyA⁺RNA-A
17 (TaKaRa), was added in each series for normalization. The fluorescent-labeled cDNA
18 prepared for hybridization was generated with an RNA Fluorescent Labeling Core Kit

1 (Takara) using 30 µg of total RNA and oligo(dT) according to the manufacturer's
2 instructions. DNA microarrays were hybridized for 10 h under coverslips with Cy5 or
3 Cy3-dCTP (Amersham)-labeled cDNA probes, washed, dried and scanned immediately in
4 an Affymetrix 428 Array Scanner (Affymetrix Instruments). Data were analyzed using
5 ImaGene software (Biodiscovery Inc.).

6

7 **5' RACE and Northern blot analysis**

8 Total RNA was isolated from Sf9 or HeLa14 cells at 48 hpi as above. 5' RACE was
9 performed using the GeneRacer core Kit (Invitrogen) according to the manufacturer's
10 instructions. Briefly, mRNAs were dephosphorylated, decapped and ligated with an adaptor
11 oligo RNA supplied in the kit. A 1st PCR was then carried out with an adaptor primer from
12 the kit and gene specific 1st PCR primers (*ie-1* and *ie-0*:
13 5'-GTCTGTTCAAGGGTTGCACAGC-3', *pe38*: 5'-GGCTGGCGCACTGTCGTCAC -3',
14 *gp64*: 5'-AGACTGGTGCCGACGCCGCC -3', *p6.9*:
15 5'-GCGTGTTCTGTA ACTTCGGCGACC-3'). A 2nd PCR was performed using an
16 adaptor-specific nested primer and gene-specific nested primers (*ie-1* and *ie-0*:
17 5'-AACTGGCCCACCACACCTTGTG-3', *pe38*: 5'-CCGTAATGCCACGTTGCGGC-3',
18 *gp64*: 5'-CGACCAGCCGCTGGCATCTTTC-3', *p6.9*:

1 5'-GGGGTCTACCCGGGCGGCGT-3'). PCR products were cloned using an AT cloning
2 pGEM-T Easy vector system (Invitrogen) and at least four cDNA were sequenced using the
3 ABI PRISM 310 Genetic Analyzer for each gene.

4 Total RNA (10µg) isolated at the time presented in the figure was denatured and separated
5 by electrophoresis in a 1% agarose gel, transferred to a nylon membrane (Hybond-N+;
6 Amersham) and hybridized with oligonucleotide probes (human β-actin: 5'-
7 ACGTCACACTTCATGATGGAGTTGAAGGTAGTTTCGTGGATGCCACAGGACTCC
8 ATGCCT-3', human 18SrRNA: 5'-CTGGACCGGCCCTGCGTACTTAGACATGTAT
9 GGCTTAATCTTTGAGACAAGCATATGGTT-3') labeled with [γ^{32} -P]ATP (Amersham)
10 using MEGALABEL (Takara). Quantitative analysis was performed with an Image Reader
11 BAS1000 (Fuji film).

12

13

14 **Results**

15 **DNA microarray analysis in mammalian cells**

16 We adopted a DNA microarray to analyze the transcripts of *AcMNPV* in the mammalian
17 cells. Cy3 or Cy5-dCTP labeled cDNA probes were simultaneously hybridized to probe
18 sequences on the microarray, and the amount of fluorescence seen with the individual dyes

1 was determined by microarray scanner. The positivity of the signals of the gene spots on
2 the DNA array chips was judged under the criteria “significantly positive: Signal mean –
3 (Background mean + 2×Background Standard deviation) > 0” (Fig. 1A). Forty-three viral
4 genes were detected in the HeLa14 cells and 14 genes in the BHK cells (Table 1). The
5 experiment was duplicated and the similar results were obtained. Twelve of these genes
6 overlapped in the two cell types, as confirmed by nested RT-PCR using gene-specific
7 primers (data not shown).

8

9 **Fidelity in transcription**

10 The fidelity of transcription initiation for the *AcMNPV* genes in HeLa14 cells was then
11 analyzed by 5'RACE. First of all, transcription initiation sites of *ie-0*, *ie-1*, *gp64* and *pe38*
12 in Sf9 cells were determined at 48 hpi (Fig. 2). The transcription start site of *ie-1* in Sf9
13 cells was located in a CAGT motif (early gene motif) located 77 nts upstream of the
14 translation start codon, consistent with that reported previously (25). In HeLa14 cells, *ie-1*
15 was also transcribed from this site (Fig. 2). *ie-0* and *gp64* of *AcMNPV* have both a CAGT
16 early motif (16, 9) and a TAAG late motif (4). 5' RACE using mRNA purified from Sf9
17 cells at 48 hpi demonstrated that both genes were transcribed from the TAAG motif as
18 previously reported (16, 7, 34) while the transcription start sites of these genes in HeLa14

1 cells were located in the CAGT early motif (Fig. 2). Furthermore, sequencing of the cDNA
2 synthesized against the 5' terminal region of *ie-0* transcripts, which is known as the only
3 baculoviral transcript produced by splicing (11), revealed that the intron sequence (nts
4 122946 to 127149, GB No., L22858) was precisely spliced out in HeLa14 cells. On the
5 other hand, the transcription of *pe38* was rather complicated. At 48 hpi, two transcription
6 initiation sites for *AcMNPV pe38* were elucidated in Sf9 cells, one in a TAAG motif
7 located 4 nts upstream of the early CAGT motif and another located 452 nts downstream of
8 the early motif (Fig. 2A) (20). This feature of the transcription, ie an internal transcription
9 initiation site, corresponded with that observed for the *Orgyia pseudotsugata* NPV
10 (*OpNPV*) *p34* gene, a homologue of *AcMNPV pe38* (31). The internal transcription
11 initiation site generates a small mRNA with the capacity to code for a protein with a
12 calculated molecular mass of 20 k both in *OpNPV* (31) and in *AcMNPV*. The transcription
13 of *pe38* in HeLa14 cells started at several sites which were not located in the CAGT motif
14 (Fig. 2). The transcription start site of the late gene *p6.9* in HeLa14 cells was located 99 nts
15 downstream of the late TAAG motif (Fig. 2).

16

17 **Upregulation of β -actin expression in mammalian cells inoculated with *AcMNPV***

18 As described elsewhere (32), the NPV DNA microarray contains human β -actin and λ

1 phage sequences as internal controls for normalization between chips. Interestingly, the
2 relative intensity of the signal for *β-actin* on the DNA array was clearly increased in BHK
3 cells (Fig. 1) and moderately increased in HeLa14 cells (data not shown) inoculated with
4 *AcMNPV*. We then carried out Northern blot hybridization using total RNA isolated from
5 HeLa14 cells to analyze the kinetics of the transcription of *β-actin* (Fig. 3). The amount of
6 transcript from *β-actin* in HeLa14 inoculated with *AcMNPV* increased until 6 hpi, reached
7 a level about 2.5-fold that of the control and then rapidly decreased, while the amount of
8 transcript in the control cells gradually decreased until 24 hpi. A similar profile for the
9 transcription of *β-actin* was obtained from BHK cells (data not shown). UV-inactivated
10 *AcMNPV* also induced upregulation of *β-actin* expression at a level comparable to that
11 induced by non-irradiated *AcMNPV* at 6 hpi (Fig. 3B lanes 1-3).

12

13

14 **Discussion**

15 The host range of baculoviruses is restricted to arthropods including insects and crustaceans.
16 The host specificity of *AcMNPV* was originally thought to be restricted to cells derived
17 from insects. However, several groups recently reported that *AcMNPV* can transfer and
18 express foreign genes under the regulation of appropriate eukaryotic promoters in several

1 types of mammalian cells and animal models (18). This observation led us to consider its
2 use in gene therapy given its low cytotoxicity in mammalian cells even at a high MOI, an
3 inherent incapability to replicate in mammalian cells, and the absence of preexisting
4 antibodies against baculoviruses in animals. On the other hand, Beck *et al.* reported that
5 *AcMNPV* infection repressed phenobarbital-mediated gene induction and stimulated
6 TNF-alpha, IL-1 alpha, and IL-1 beta expression in primary cultures of rat hepatocytes (5),
7 indicating that mammalian cells could be physiologically influenced by the experimental
8 attack with *AcMNPV*. More recently, *AcMNPV* was shown to induce an innate immunity
9 that confers protection from a lethal challenge of influenza virus in mice through the
10 recognition of the baculovirus genomic DNA by a Toll-like receptor (TLR) molecule (1, 3).
11 These observations are thus not in contradiction to previous observations that *AcMNPV*
12 genes were not transcribed in HeLa cells inoculated with *AcMNPV* at a MOI of 100 PFU
13 per cell (MOI of 100) at 24 hpi or after several passages (28). In contrast, we clearly
14 detected the viral transcripts in mammalian cells inoculated with *AcMNPV* at a lower MOI
15 (MOI of 30) using a DNA microarray and 5'RACE technologies. This discrepancy might
16 be due to the difference in the sensitivity of the detection methods used in the two
17 experiments.

18 5'RACE analysis revealed that all genes examined in this study, except *pe38* and *p6.9*,

1 were transcribed from an early transcription start site motif (CAGT) preceded by a
2 TATA-motif 30 nts upstream in HeLa14 cells (Fig. 2). The CAGT motif is a part of the
3 eukaryotic transcription initiation site sequence (INR; YYANT/AYY) (27) and the early
4 promoters for these viral genes with a typical eukaryotic RNA polymerase II promoter
5 structure (a conventional TATA motif and an INR) are functional in HeLa14 cells.
6 Furthermore, the transcripts from *ie-0* were observed to be precisely spliced (11) in HeLa14
7 cells, showing the compatibility of the splicing machinery between mammalian cells and
8 insect cells. These observations suggested that there was no critical disadvantage in the
9 transcription of these genes in mammalian cells. A late gene, *p6.9* was also transcribed
10 from 25 nts downstream of a TATA-like motif in the HeLa14 cells. However, the
11 transcription initiation site was located in the coding sequence (Fig. 2). Four transcription
12 initiation sites (nucleotide positions +112, +454, +552 and +575) were identified for *pe38*
13 in HeLa14 cells and none of them corresponded with the start sites in Sf9 cells. The most
14 upstream site (+112) and the second site (+454) were located in the INR-like sequences
15 CCGCAGA and CCATTGT, respectively, which were also accompanied by a TATA-motif
16 25 nts upstream (Fig. 2A). The sequences of surrounding the other two sites showed no
17 significant similarity to INR but were preceded by sequences with some similarity with the
18 TATA-motif or TFIIB recognition element G/CG/CG/ACGCC (BRE) (17, 26) 30 nts

1 upstream (Fig. 2B). However, we could not obtain evidence for the transcript from a CAGT
2 motif located 4 nts downstream of the transcription start site +1 for *pe38* in HeLa14 cells
3 though it was also accompanied by a TATA-motif 30 nts upstream. This suggested that the
4 typical eukaryotic RNA polymerase II promoter structure was not sufficient to initiate
5 transcription in the *AcMNPV* genome.

6 We happened to observe the upregulation of *β-actin* expression in *AcMNPV*-inoculated
7 mammalian cells in an analysis using a NPV DNA microarray containing a human *β-actin*
8 DNA spot as a control. Actin is essential for the transport of NPV to the nucleus in insect
9 and mammalian cells (29) and is also necessary not only for constitution of the virogenic
10 stroma but also for budding of the viruses in insect cells (6). In insect cells, *AcMNPV*
11 infection induces overexpression of cellular actin and it inhibits the polyhedron synthesis and
12 polyhedra formation, although factors stimulate actin expression are not clear (30). In order
13 to investigate if the upregulation is related to the viral transcripts in HeLa14 cells, effects of
14 UV-inactivated viruses on *β-actin* expression were examined. UV-inactivated viruses
15 which were expected to be impaired in the fusion capability of envelope protein by the
16 denaturation of GP64 (1) were able to induce an upregulation of *β-actin* expression
17 compared to untreated control viruses, suggesting that the upregulation was caused by
18 events occurring before the internalization of viral components. Thus, the significance of

1 the *AcMNPV* transcript to the physiology of mammalian cells remains to be solved.

2 In conclusion, the results of our investigation provided evidence that *AcMNPV* is
3 capable of expressing some viral genes at least at the transcription level in mammalian cells
4 through the usual pathway of infection. These results emphasize the significance of
5 studying the molecular details of baculovirus-mammalian cell interactions to reinforce the
6 inability of *AcMNPV* to replicate in mammalian cells for facilitating the use of
7 baculoviruses in the agro-biological, pharmaceutical and medical fields.

8

9

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15 **Figure legends**

16

17 **Fig. 1 (A)** DNA microarray analysis using Cy5-labeled cDNA probes. The cDNA probes
18 were synthesized from total RNA extracted from mock-infected cells (**left**) or

1 *AcMNPV*-infected BHK cells (at a MOI of 30) (**right**). Daggers indicate the positions of
2 the spots for β -*actin*. Asterisks indicate the internal control, λ polyA.

3

4 **Fig. 2** Mapping of the 5' ends of the transcripts for *ie-1* and *ie-0*, *gp64*, and *pe38* in Sf9 and
5 HeLa14 cells at 48 hpi. (A) Schematic representation of the gene structure for *ie-1* and *ie-0*,
6 *gp64*, and *pe38*. The open box shows the open reading frame. Arrows on the diagram for
7 each gene indicate the initiation sites in Sf9 cells at the early (E) or late (L) stage and in
8 HeLa14 cells (M), respectively. (B) Sequences around the transcription initiation sites in
9 Sf9 cells and HeLa14 cells. The arrow shows the transcription initiation site and the
10 number in parenthesis indicates the nucleotide position relative to the transcription
11 initiation site in Sf9 cells (+1) at the early stage (for *ie0* (16), *ie1* (25), *gp64* (9), and *pe38*
12 (20)) or the late stage (for *p6.9* (this paper)). The CAGT early motif and the TAAG late
13 motif are underlined. Bold type indicates TATA-box like and BRE-like sequences.

14

15 **Fig. 3** Northern hybridization assays using ³²P-labeled β -*actin* and 18SrRNA cDNA probes.
16 (A) Northern hybridization was performed using total RNA purified from
17 *AcMNPV*-infected HeLa14 cells at the times shown in the panel. Kinetics of the expression
18 of β -*actin* is presented as a sequential line graph below the autoradiograph and the values

1 are shown as the ratio of β -actin to 18SrRNA on the basis of the signal intensities obtained
2 from the Northern hybridization. Circles and boxes in the graph indicate the kinetics for
3 *AcMNPV*-infected and mock-infected cells, respectively. **(B)** Northern hybridization assay
4 using total RNA from Mock-infected (1), *AcMNPV*-infected (MOI of 30) (2) and
5 UV-inactivated virus-infected (3) cells. The bar graph below the autoradiograph shows the
6 ratio of hybridization signal for β -actin to that for 18SrRNA.

7

Table 1 Viral genes judged as “positive (transcribed)” in DNA microarray analysis

Cells	Promoter	Genes					
HeLa14	E	<u>orf149</u>	<i>egt</i>	<i>ie-1</i>			
	E&L	<u>pe38</u>	<u>ie-2</u>	<u>ie-0</u>	<i>gp64</i>	<i>me53</i>	
	L	<u>orf91</u>	<u>he65</u>	<u>p6.9</u>	<u>orf142</u>	<i>ptp</i>	<i>odv-e56</i>
		<i>ets</i>	<i>orf19</i>	<i>vp80</i>	<i>arif-1</i>	<i>orf38</i>	<i>orf120</i>
		<i>orf4</i>	<i>orf66</i>	<i>orf102</i>	<i>orf92</i>	<i>orf75</i>	<i>orf78</i>
		<i>orf13</i>	<i>orf73</i>	<i>orf108</i>	<i>p35</i>		
	The others	<u>orf140</u>	<u>orf152</u>	<u>odv-e26</u>	<i>pp34</i>	<i>dbp</i>	<i>lef-6</i>
		<i>p47</i>	<i>lef-3</i>	<i>orf29</i>	<i>orf41</i>	<i>orf97</i>	<i>orf45</i>
<i>orf117</i>							
BHK	E	<u>orf149</u>					
	E&L	<u>pe38</u>	<u>ie-2</u>	<u>ie-0</u>	<i>gp64</i>		
	L	<u>orf91</u>	<u>he65</u>	<u>p6.9</u>	<u>orf142</u>	<i>hisp</i>	
	The others	<u>orf140</u>	<u>orf152</u>	<u>odv-e26</u>	<i>94k</i>		

* E or L indicates the presence of the following promoter motifs. E, early promoter motif (TATA box followed by CAGT motif); L, late promoter motif ((A/T/G)TAAG). Underlines indicate the genes which detected both in HeLa14 and in BHK cells.

Fig.1

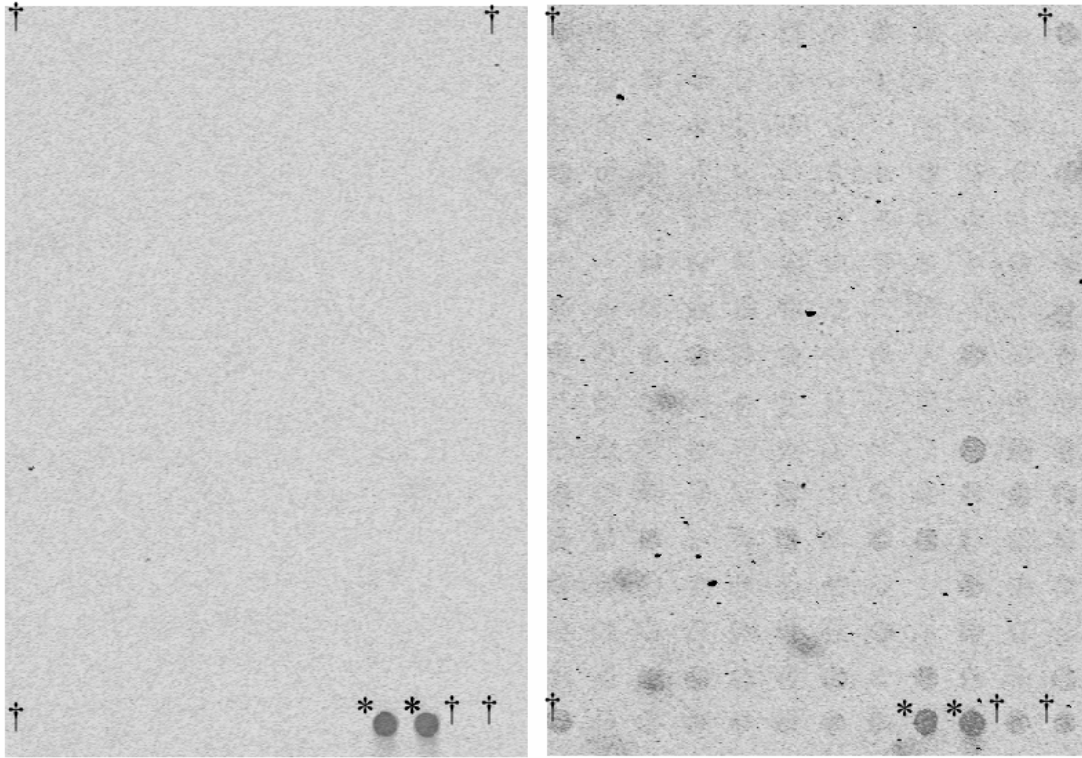


Fig. 2

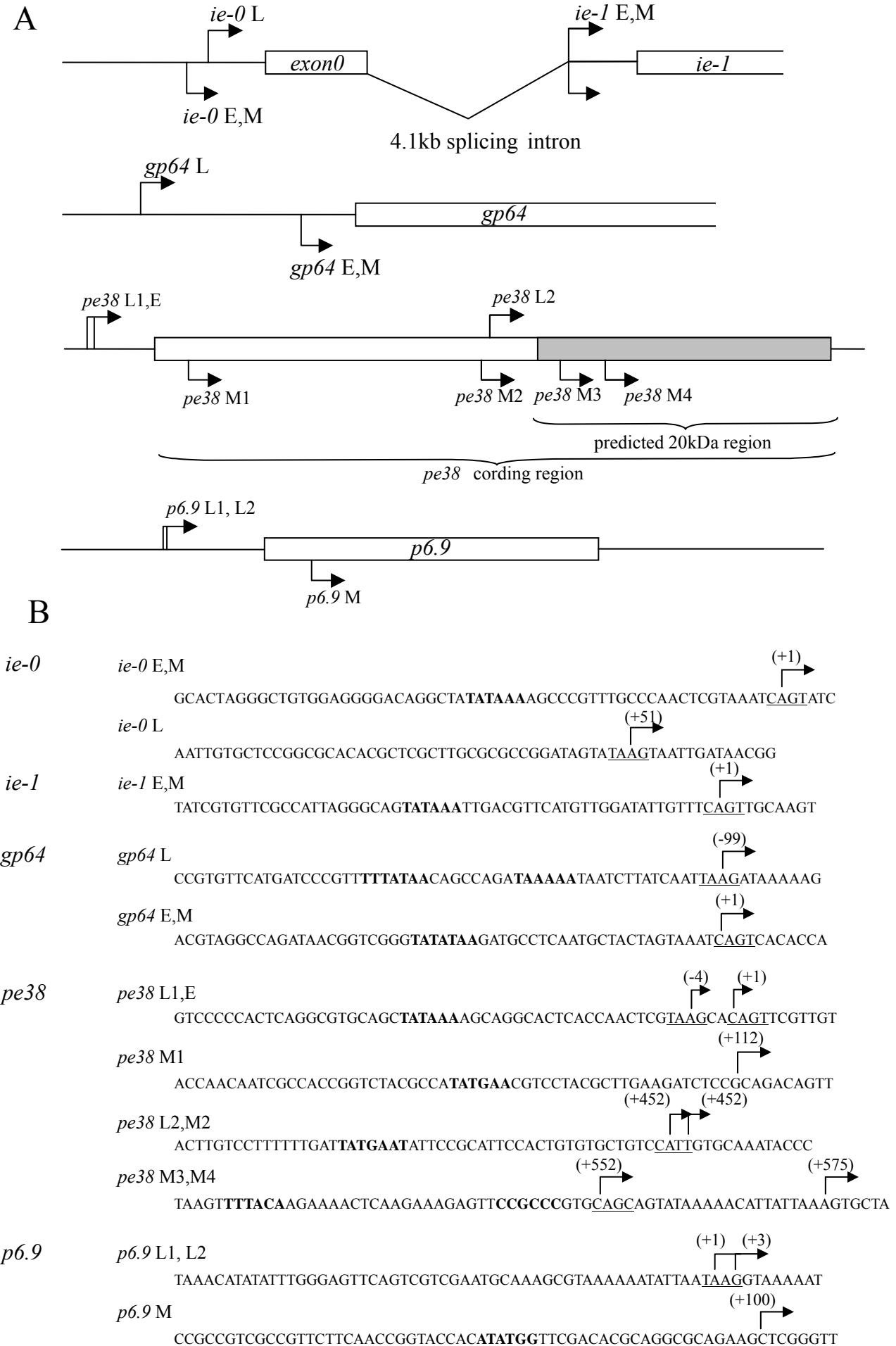


Fig.3

