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Ars Insulator Identified in Sea Urchin Possesses an Activity to Ensure the Transgene Expression in Mouse Cells

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Sea urchin arylsulfatase (Ars) gene locus has features of an insulator, *i.e.*, blocking of enhancer and promoter interaction, and protection of a transgene against positional effects [Akasaka *et al.* (1999) *Cell. Mol. Biol.* 45, 555–565]. To examine the effect of Ars insulator on long-term expression of a transgene, the insulator was inserted into LTR of retrovirus vector harboring hrGFP gene as a reporter, and then introduced into mouse myoblast cells. The isolated clones transduced with the reporter gene with or without Ars insulator were cultured for more than 20 wk in the absence of a selection reagent, and the expression of hrGFP was periodically determined. Expression of hrGFP in four clones transduced with the reporter gene without Ars insulator was completely silenced after 20 wk of culture. On the other hand, hrGFP was expressed in all clones with Ars insulator inserted in one of the two different orientations. Histone H3 deacetylation and DNA methylation of the 5′LTR promoter region, signs for heterochromatin and silencing, were suppressed in the clones that were expressing hrGFP. Ars insulator is effective in maintaining a transgene in mouse cells in an orientation-dependent manner, and will be a useful tool to ensure stable expression of a transgene.

Key words: insulator, retrovirus vector, sea urchin, transgene expression.

Abbreviations: Ars, arylsulfatase; ChIP, chromatin immuno-precipitation; HPRT, hypoxanthin-guanine phosphoribosyltransferase; IRES, internal ribosome entry site.

Chromatin insulator is a DNA sequence that serves as a boundary element between differentially regulated genes. Various insulators have been found in *Drosophila* and vertebrates, including chicken, mouse and man (*1–4*). These insulators have two conserved properties (*5*). One is an enhancer-blocking activity: an insulator blocks enhancer and promoter interaction when placed between them. The other is protection from positional effects. A promoter/reporter cassette surrounded by insulators is expected to be isolated from the local chromosomal environment, and therefore to be protected from positional effects. The most extensively investigated vertebrate insulator, cHS4, is derived from the chicken β-globin gene locus control region (*1*, *6*, *7–11*). Previous reports have shown that cHS4 insulator protects a transgene from position-effect variegation when introduced into Moloney murine leukemia virus–based retroviral vectors (*12*, *13*). Since the cHS4 fragment is active in restricted cell types (*13*) an alternative insulator is necessary to propagate the use of the insulator in various cell types and tissues.

It is crucial for the production of transgenic animals and gene therapy to ensure stable expression of an ectopically introduced gene, a transgene. For these purposes, many vectors have been developed, among which retroviral vectors have been extensively utilized as vehicles. Although these vectors assure efficient gene delivery into the host genome, expression from these vectors depends on the site of integration in the host genome. This phenomenon, so-called position-effect variegation, is one of the major defects of these vectors (*14*). Another problem relevant to these vectors is the silencing of a transgene that had been highly transcriptionally active. This transgene silencing has been observed both *in vitro* and *in vivo* across various cell types and species (*6*, *15*, *16*). Accumulating evidence suggests that epigenetic alterations of the transgene, such as DNA methylation and/or histone modifications, are likely causes (*6*, *15–17*).

The 573-bp fragment of sea urchin arylsulfatase (Ars) gene locus is reported to have typical features of an insulator, such as blocking of enhancer and promoter interaction in sea urchin embryos, and position-effect protection in HeLa cells (*18*). Since Ars insulator works in various cell types and across species, it has been suggested that this non vertebrate-derived insulator may serve as a universal insulator (*18–20*). Recently, it has been shown that Ars insulator protects lenti viral vector from silencing (*21*). However, since lentivirus vector is more resistant to silencing than a Moloney murine leukemia virus (MoMLV)–based vector, MoMLV vector is suitable to study the anti-silencing mechanism of the insulator. Therefore, we further developed a Moloney murine retrovirus–based vector that easily becomes silent after long-term culture, and

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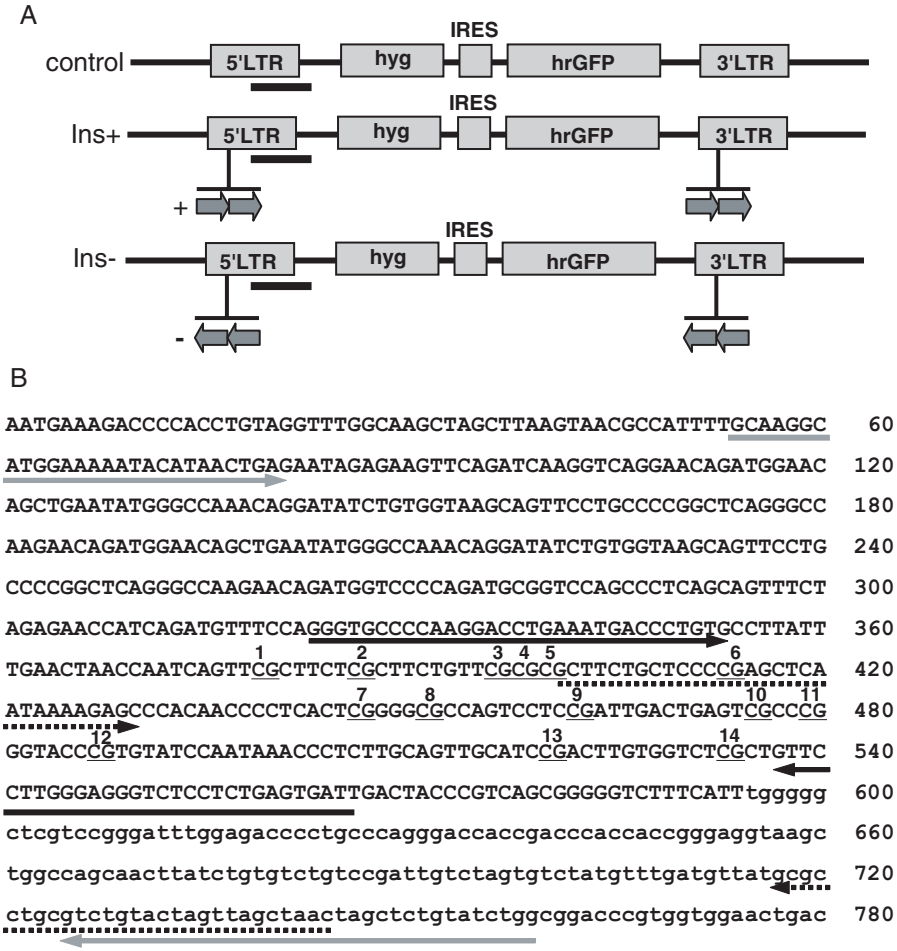

Fig. 1. **Schematic illustration of the reporter genes integrated into genome.** A: A cassette of hygomycin resistant gene (hyg) and IRES-hrGFP was placed between 5′ and 3′LTRs. Two copies of Ars insulator in tandem were inserted into 5′ and 3′ LTRs when the reporter gene was integrated into genome. The signs + (Ins+) and – (Ins–) indicate the orientations of Ars insulator that block an enhancer effect from the 3′ downstream and 5′ upstream directions, respectively. The underlines including the 5′LTR are the sequence shown in B. B: The 5′LTR (upper cases) and its 3′ adjacent sequences (lower cases) are shown. The primers for ChIP assay are indicated by dotted arrows, and those for bisulfite analysis are indicated by gray (1st PCR) and black (2nd PCR) arrows. The underlined CG sites with numbers are the sites analyzed with regard to the methylation state.

inserted into it an Ars insulator to examine its anti-silencing activity.

MATERIALS AND METHODS

Construction of Reporter Gene—The vector pRCV/HygIREShrGFP with or without Ars insulator was constructed from Moloney murine retrovirus vector pLNSX (accession number M28246). The neomycin phosphotransferase gene driven by SV40 early promoter in pLNSX was displaced with a hygromycin resistant gene (hyg) connected to an hrGFP gene with an internal ribosome entry site (IRES) between them. Hyg and IRES-hrGFP were prepared by PCR amplification using pRevTRE (Clontech) and pIRES-hrGFP-1a (Stratagene) as templates, respectively. Two copies of Ars insulator in tandem were inserted into the *Nhe*I site of the 3′LTR in two different orientations, + and – (Fig. 1). The constructed plasmids and pcDNA-VSV-G were co-transfected into gag-pol-expressing cells to produce packaged virus. Culture supernatant was collected and used for transduction.

Transduction and Cell Culture—Murine myoblast C2C12 cells were maintained at 37°C under 5% CO_2 atmosphere in DMEM supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. C2C12 cells were transduced with viral vectors added with 4 μg/ml polybrene at a multiplicity of infection (MOI) of 0.1 for 16 h. After 48 h of culture, cells were transferred onto 96-well plates at a density of one cell per well and cultured in the presence of 0.4 mg/ml of hygromycin B. Independent clones were picked up from each well in which a single colony was identified under the microscope.

FACS—Isolated clones were examined for expression of hrGFP with a FACSCalibur flowcytometer using CellQuest software (Becton and Dickinson). Among the isolated clones transduced with the reporter genes without and with Ars insulator in + and – orientations, 10 each of the clones that expressed hrGFP in high level were randomly chosen and further cultured in the absence of hygromycin B. The expressed hrGFP fluorescence was monitored at 2-wk intervals under identical conditions.

Chromatin Immuno-Precipitation (ChIP)—ChIP assay was performed basically as described elsewhere (*21*). Briefly, C2C12 cells (5×10^5 cells) were fixed with formaldehyde and then sonicated to obtain soluble chromatin. Soluble chromatin was immuno-precipitated with anti–acetylated histone H3 antibody (Upstate Biotechnology) overnight at 4°C. Immuno-precipitates were collected with 50% protein A and G-Sepharose slurry preabsorbed with 0.1 mg/ml sonicated salmon sperm DNA. Purified DNA was subjected to PCR reactions using recombinant Taq polymerase (Toyobo) and primer sets specific for the 5′LTR promoter, and promoters of hypoxanthin-guanine

phosphoribosyltransferase (HPRT) and albumin genes. The PCR primer sequences are as follows:

5′LTR sense, 5′GCTTCTGCTCCCCGAGCTCAATAAAAGAGC3′, and antisense, 5′GCGCCTGCGTCTGTACTAGTTAGCTAAC3′.

HPRT sense, 5′ACCTAGTCAGATAAGAGTTCCGGAACTGCC3′, and antisense, 5′CGGAAAGCAGTGAGGTAAGCCCAACGC3′.

Albumin sense, 5′TTGGGATGAACAACCTATGCAATTCAGTTC3′, and antisense, 5′ACCCCATTACAAAATCATACCATCTTTGCC3′.

The amplification reactions were as follows: for the 5′LTR, 35 cycles of denaturation at 94°C for 10 s, annealing at 65°C for 5 s, extension at 72°C for 30 s; for the HPRT gene, 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s; for the albumin gene, 30 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, extension at 72°C for 30 s. In each PCR reaction, denaturation at 94°C for 2 min and extension at 72°C for 2 min were performed before and after the multiple cycles. PCR products were separated on agarose gel, and the DNA bands were stained with ethidium bromide.

DNA Methylation Analysis—Genomic DNA was prepared by Wizard SV genomic DNA purification system (Promega), and the purified DNA was modified with sodium bisulfite using EZ DNA methylation kit (Zymo Research, CA, USA). The 5′LTR of the modified DNA thus prepared was PCR amplified with recombinant Taq polymerase using the following primers, of which the sites are illustrated in Fig. 1B.

sense 1, 5′GTAAGGTATGGAAAAATATATAATTGAGAATAGAG3′;
antisense 1, 5′CCAAATACAAAACTAATTAACTAACTAATACAAAC3′;
sense 2, 5′GGGTGTTTTAAGGATTTGAAATGATTTTGTG3′;
antisense 2, 5′AATCACTCAAAAAAAACCCTCCCAAAAAAC3′.

The first amplification reaction using sense 1 and antisense 1 primers comprised denaturation at 94°C for 2 min, and then 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s. Extension at 72°C for 2 min was performed after the multiple cycles. The amplified mixture was further amplified with sense 2 and antisense 2 primers by denaturation at 94°C for 2 min, and then 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s. The amplified DNA was subcloned into pBluescript II, and more than twelve clones were sequenced for each amplified fragment.

RESULTS

Effect of Ars Insulator on the Maintenance of hrGFP Expression in Mouse Myoblast Cells—It was reported that a transgene expression is effectively protected from silencing by the surrounding two units of chicken β-globin insulator, cHS4 (*6*). Each unit comprises two copies of cHS4 in tandem. Therefore, in the present study, to determine the effect of Ars insulator, two copies in tandem of Ars insulator in two different orientations were inserted into the LTR (Fig. 1). The host cells chosen to examine the effectiveness of the Ars insulator were the established myogenic progenitor cells of C2C12. C2C12 cells were originally established from the satellite progenitor cells of skeletal muscle cells (*22*), and thus are not transformed. Such nontransformed cells are suitable to mimic the *in vivo* state. Ten each of the transduced and isolated clones of C2C12 cells that carried a hrGFP-reporter gene with or without Ars insulator were randomly chosen and cultured for more than 20 wk in the absence of hygromycin B, which was used for the selection of the clones. The doubling times of all the isolated clones were in a similar range, indicating that the integration of a single transgene did not severely affect the proliferation of the host cells (data not shown). The transgene with or without Ars insulator inserted into the genome did not seem to affect the fate of cells drastically, as the histone methylation states in the promoter regions of endogenous HPRT and albumin genes were not affected (see Fig. 4). The expression level of hrGFP in each clone was determined by FACS analysis, and the average hrGFP fluorescence was monitored periodically and plotted (Fig. 2). When no Ars insulator was inserted into the reporter gene, the expression of hrGFP in four clones was completely silenced (4c, 14c, 24c, and 32c) after 20 wk of culture in the absence of hygromycin B (Figs. 2A and 3A, and Table 1). The completely silenced clones were defined as those with an average fluorescence of less than 10.1, which is the average value plus 3 SD of the value obtained for the parent C2C12 cells (Table 1). In addition, the expression of hrGFP in one clone, 37c, decreased to less than 20% of the initial level after 20 wk of culture. This clone showed a mixture of a major population of silent cells and a minor population of the cells expressing hrGFP (Fig. 3A). Unexpectedly, five clones, namely half of the clones examined, escaped silencing of the hrGFP gene, of which the expression level was more than 30% of the initial level.

At the same time, we examined the reporter gene inserted with two copies each of Ars insulator into the 5′ and 3′ LTRs in two different orientations (Fig. 1A). In clones designated with "+," the Ars insulator was inserted to block the external enhancer effect from downstream of the reporter gene; and in clone designated with "–," it was inserted to block the enhancer effect from the upstream. Of the + clones, the expression of hrGFP in two clones (1+ and 12+) was completely silenced, and that in another clone decreased to less than 20% of the initial level (2/28+) after 20 wk of culture (Figs. 2B and 3B, and Table 1). Clone 2/28+ showed a shift in the fluorescence intensity of hrGFP to a lower level without a split in the cell population (Fig. 3B). The remaining seven clones kept more than 40% of the initial hrGFP expression level.

When the Ars insulator was inserted into the 5′ and 3′ LTRs in the – orientation, the maintenance of hrGFP expression was prominent. None of the clones showed completely silenced expression of hrGFP, which was above 20% of the initial level after more than 20 wk of culture (Fig. 2C and Table 1).

In the absence of Ars insulator, four out of ten clones were completely silenced. On the other hand, two and none out of ten clones were silenced in the clones transduced with the transgene containing Ars insulator in + and – orientations, respectively. To examine whether the Ars

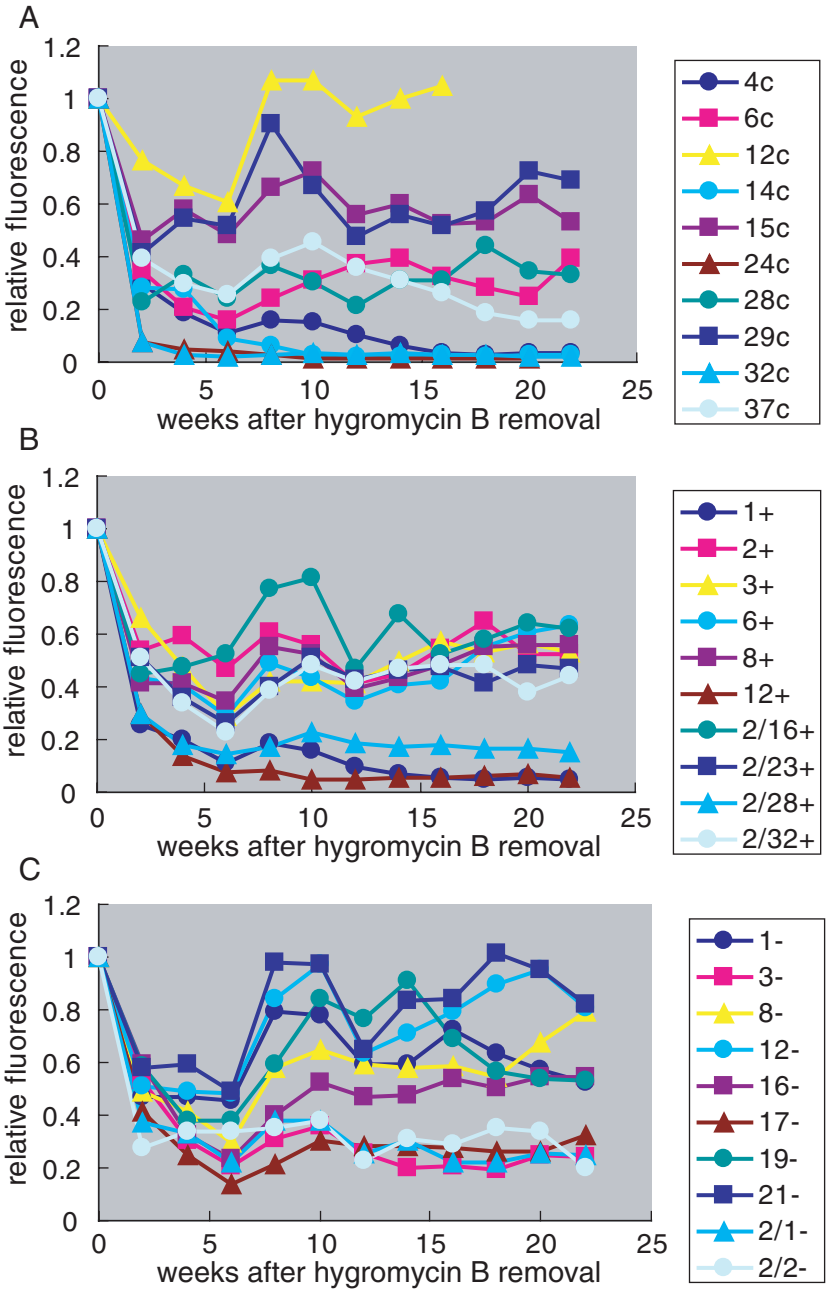

Fig. 2. **Expression of hrGFP after hygromycin B removal in isolated clones.** A: Ten transduced clones with the reporter gene without Ars insulator were cultured in the absence of hygromycin B, and the expression of hrGFP (relative fluorescence) was monitored at 2-wk intervals with FACS. The average fluorescence normalized to that before the removal of hygromycin B is plotted. B and C: Expression of hrGFP of the 10 clones each that were transduced with the reporter gene inserted the Ars insulator in + (B) and – (C) orientations were monitored as in A.

insulator insertion positively affected the maintenance of expression of transgene, Fisher's exact probability test was performed. The probabilities of the hypotheses that "the Ars insulator was not effective for the anti-silencing" for the Ars insulator in the + and – directions were calculated to be 0.314 and 0.043, respectively. The probability of 0.043 is significantly low leading to the conclusion that the Ars insulator inserted in the – direction was effective in maintaining expression of transgene. On the other hand, the Ars insulator in the + direction was not effective, although it showed a tendency to protect against a wave of gene silencing.

Histone Acetylation Levels at the 5′LTR Region in the Clones before and after Long-Term Culture—It has been established that gene silencing of a transgene parallels the deacetylation of histones (*16*, *17*, *23*). To see if the deacetylation of histones coincided with the silencing of hrGFP, ChIP with the antibody against acetylated histone H3 was performed, and then the 5′LTR region of the precipitated DNA was PCR amplified (Fig. 1). As references, the primer sets for amplification of the promoters of HPRT and albumin genes were used as positive and negative controls, respectively. The HPRT gene is a housekeeping gene and is expressed ubiquitously, and the albumin gene is a tissue-specific gene and is not expressed in myoblast cells such as C2C12 cells. The 5′LTR of the reporter gene integrated into the genome of the cloned cells that were actively expressing hrGFP was expected to be pulled down with anti-acetylated histone H3 antibody. Actually, the 5′LTR in all the clones that were actively expressing

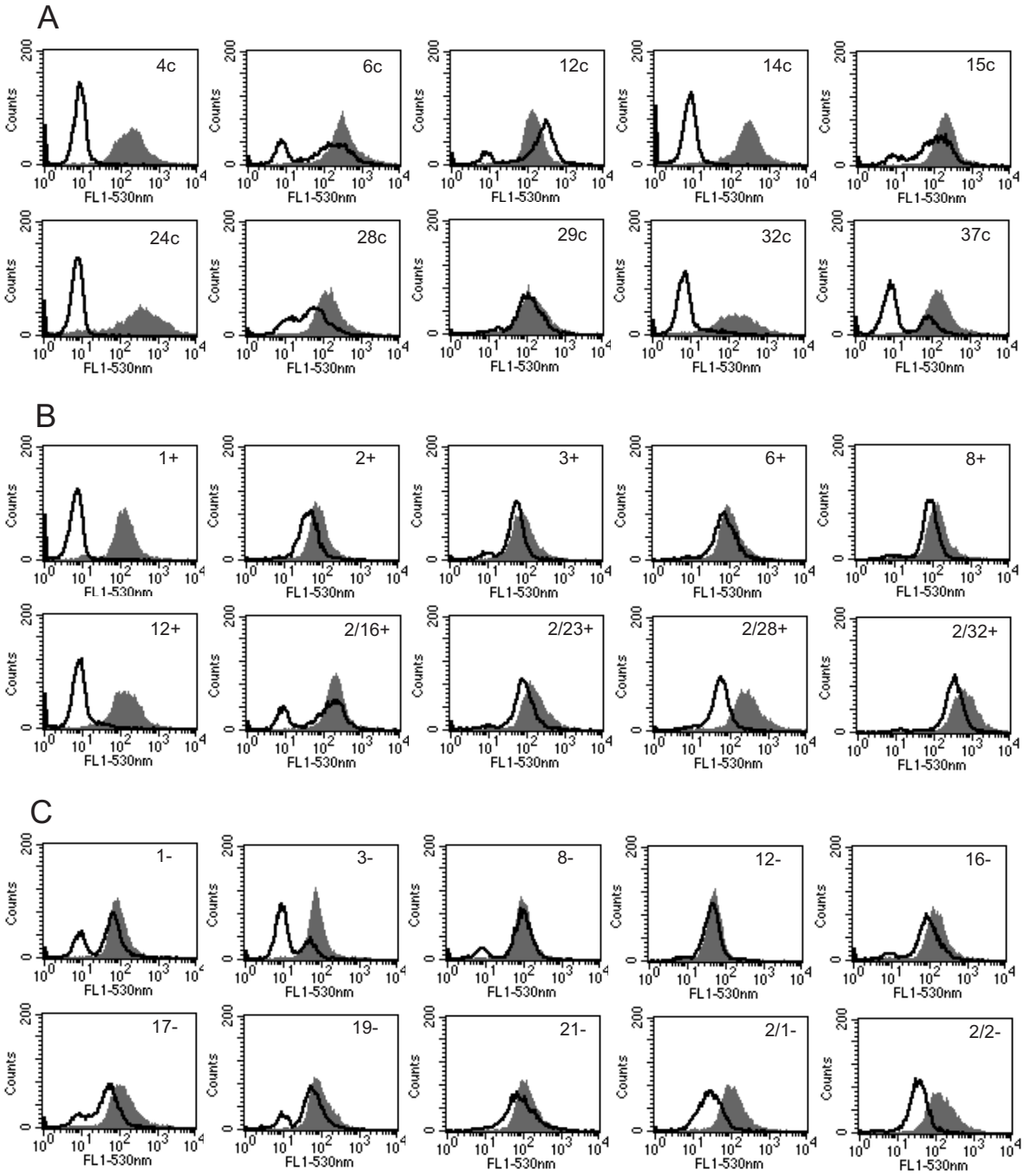

Fig. 3. **Expression of hrGFP in isolated clones before and after long-term culture in the absence of hygromycin B.** Histograms of hrGFP expression (FL) in the clones transduced with the reporter gene without Ars insulator (A), inserted with Ars insulator in + (B) and – (C) orientations are shown. Before (gray area) and after 21 to 23 wk of culture (solid line) in the absence of hygromycin B are overlaid in each figure.

hrGFP before the removal of hygromycin B was immunoprecipitaed with the antibody (Fig. 4).

Of the clones transduced with the reporter gene without Ars insulator, the 5′LTR region in five clones that maintained the hrGFP expression (6c, 12c, 15c, 28c, and 29c) was positively immuno-precipitated, indicating that histone H3 at the promoter region was acetylated. On the other hand, the 5′LTR in the clones in which the expression was silenced (4c, 14c, and 32c) were not precipitated, that is, histone H3 at the 5′LTR promoter region of these clones was deacetylated. Contrarily, two clones showed exceptional results. In clone 24c, of which hrGFP was silenced, the 5′LTR was immuno-precipitated; and in clone 37c, of which hrGFP expression was maintained, the 5′LTR was not immuno-precipitated with anti-acetylated histone H3 antibody. Since the hrGFP expression level was low in

Table 1. **Fluorescence of hrGFP, and histone acetylation at and DNA methylation of the 5′LTR region in isolated clones**. Average hrGFP fluorescence of the isolated clones without (c) or with (+ and –) Ars insulator was determined before (0 week) and after more than 20 wk (>20 wk) of culture in the absence of hygromycin B. The average fluorescence of hrGFP after more than 20 wk of culture was normalized to that at time 0 (ratio). Acetylation levels of histone H3 in the 5′LTR region were judged from the PCR shown in Fig. 4. Percentages of DNA methylation at the 5′LTR were calculated from Fig. 5.

Clone	hrGFP[a]			Acetylated H3	DNA methylation ratio
	0 week	>20 weeks	(%)		
4c	236	8	0.03	–	86.3
6c	406	160	0.39	+	17.3
12c	147	229	1.56	+	10.1
14c	338	8	0.02	–	71.3
15c	226	120	0.53	+	1.8
24c	556	7	0.01	+	38.5
28c	179	59	0.33	+	0.0
29c	183	126	0.69	+	0.6
32c	227	10	0.04	–	17.9
37c	190	30	0.16	–	61.9
Average	269	76	0.38		30.6
SD[b]	128	79	0.48		32.0
1+	144	7	0.05	–	26.2
2+	89	47	0.53	+	0.0
3+	98	52	0.53	–	0.5
6+	125	80	0.64	+	1.2
8+	147	82	0.56	+	0.0
12+	169	10	0.06	±	9.9
2/16+	220	136	0.62	+	0.5
2/23+	187	88	0.47	+	0.0
2/28+	356	54	0.15	+	0.6
2/32+	740	327	0.44	+	0.0
Average	228	88	0.41		3.9
SD	196	92	0.23		8.4
1–	96	50	0.52	+	0.0
3–	85	20	0.24	+	55.4
8–	108	86	0.80	+	0.0
12–	53	43	0.81	+	0.0
16–	172	94	0.55	+	7.1
17–	145	47	0.32	+	0.0
19–	106	56	0.53	+	0.0
21–	137	119	0.87	+	0.0
2/1–	127	32	0.25	+	1.0
2/2–	177	36	0.20	+	0.0
Average	121	58	0.51		6.4
SD	39	31	0.25		17.4

[a]The parent C2C12cells without hrGFP-reporter gene showed the value of 6.8 ± 1.1 (average ± SD, $n = 26$). [b]SD, standard deviation.

clone 37c (Fig. 2A) and the hrGFP expression of most cells was completely silenced (Fig. 3A), it is likely that the majority of the histone H3 at the 5′LTR was deacetylated, and thus the 5′LTR was not significantly precipitated.

Unexpectedly, the 5′LTR of clone 24c, of which hrGFP expression was silenced almost completely after 23 wk of culture, was positively immuno-precipitated with anti-acetylated histone H3 antibody. To see if the timing of the silencing of hrGFP gene expression coincided with the deacetylation of histone H3 at the 5′LTR region, the clones in which the hrGFP expression was eventually silenced were subjected to ChIP assay after 3 to 6 wk of culture, at which time points the hrGFP expression of these clones was almost silenced. In all the clones examined (14c, 24c, and 32c), the 5′LTR was positively pulled down with anti-acetylated histone H3 antibody, indicating that histone H3 at the 5′LTR region was at least partly acetylated after 3 to 6 wk of culture. The gene silencing and the histone deacetylation did not occur simultaneously, but sequentially, first silencing and then deacetylation of histones. Therefore, the histone H3 at the 5′LTR in clone 24c after long-term culture could be part of the way to the complete deacetylation.

In + clones, the 5′LTR in seven out of eight clones that escaped silencing (2+, 6+, 8+, 2/16+, 2/23+, 2/28+, and 2/32+) was immuno-precipitated with anti–acetylated histone H3 antibody, while that of two clones (1+ and 12+) that were silenced failed to be immuno-precipitated (Fig. 4B). Histone H3 at the 5′LTR of clone 3+, which maintained significant hrGFP expression level, was exceptionally deacetylated. The 5′LTR in all the clones transduced with reporter gene with Ars insulator in – orientation was immuno-precipitated with anti-acetylated histone H3 antibody. The acetylation of histone was accompanied by the maintenance of hrGFP expression in the clones transduced with the Ars insulator with inserted reporter gene, with one exception, clone 3+.

DNA Methylation Levels of the 5′LTR in the Clones—It has been reported that the chicken insulator cHS4 separating the β-globin gene cluster specifically blocks the deacetylation of histones, thus, preventing the methylation of the promoter of downstream genes (*6*, *11*). To determine whether the DNA methylation of the 5′LTR was prevented by Ars insulator, the DNA methylation level was analyzed by bisulfite sequencing and compared with the levels of gene silencing and histone deacetylation.

When Ars insulator was not inserted, four clones (4c, 14c, 24c, 32c) in which hrGFP expression was completely silenced were heavily methylated in the 5′LTR after long-term culture (Fig. 5). Clone 24c, which was silenced, but in which the histone H3 in the 5′LTR was incompletely deacetylated, was also heavily methylated in the 5′LTR. In addition, clone 37c, which showed low hrGFP expression level (ratio = 0.16) and in which the histone H3 in the 5′LTR was deacetylated, was heavily methylated in the 5′LTR. Judging from these results, clone 37c can be categorized as a silenced clone. The DNA methylation level of the 5′LTR strongly correlated with the silencing of hrGFP expression. Interestingly, clones 6c and 12c, which maintained the hrGFP expression even after long-term culture, were also significantly methylated in the 5′LTR.

When Ars insulator was inserted into the LTR in + orientation, only two clones, 1+ and 12+, that were silenced after long-term culture were significantly methylated. Interestingly, however, the density of methylation in the 5′LTR of these clones was sparse compared to those clones without Ars insulator. The remaining eight clones that actively expressed hrGFP were completely unmethylated in the 5′LTR. Clone 3+, which showed a significant hrGFP expression level and deacetylation of histone H3 in the 5′LTR, was completely demethylated in the 5′LTR after long-term culture. This suggests that gene silencing,

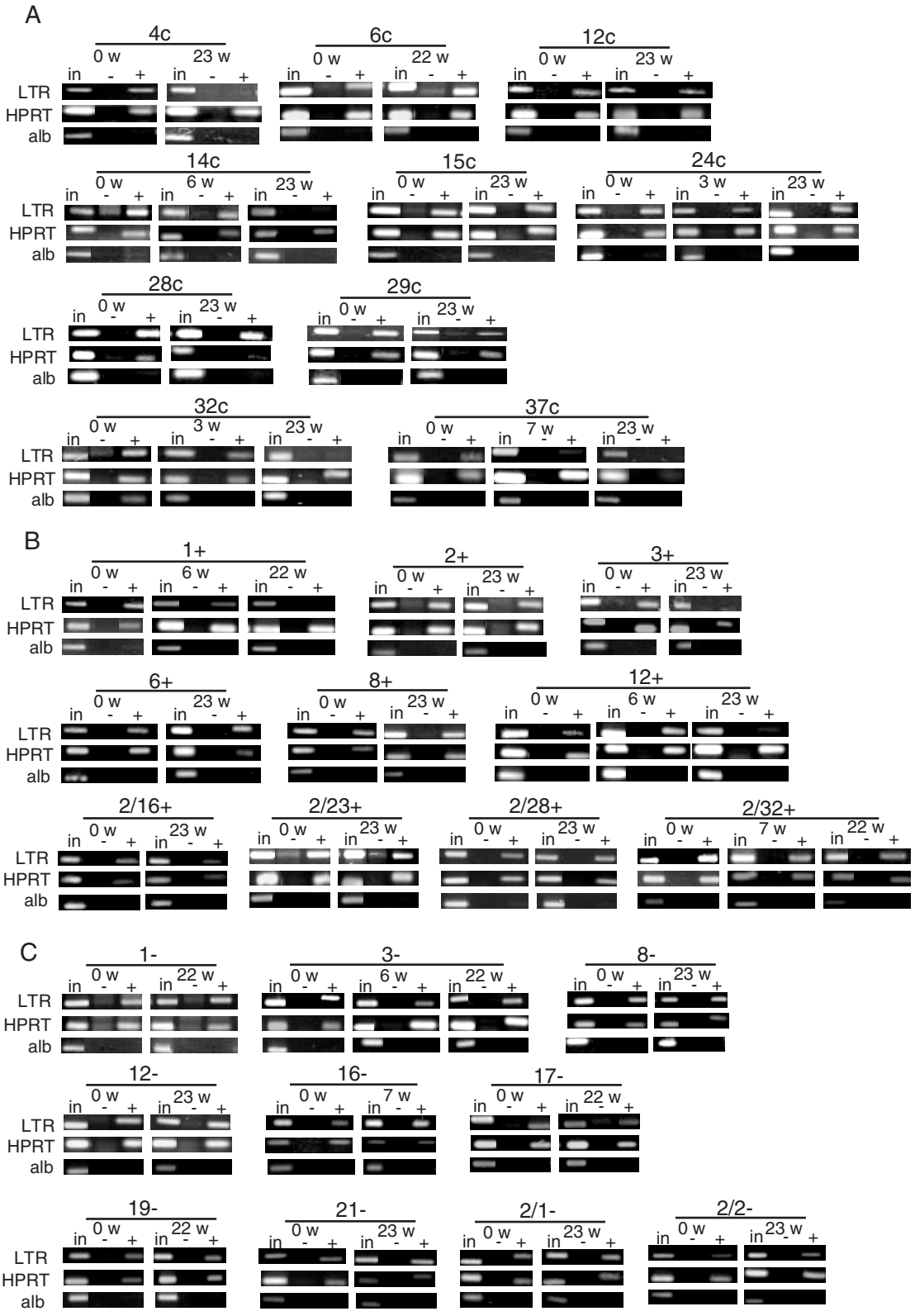

Fig. 4. **ChIP of acetylated histone H3 and PCR for the 5′LTR of the isolated clones after long-term culture.** The clones transduced with the reporter gene without Ars insulator (A), inserted with Ars insulator in + (B) and – (C) orientations were subjected to ChIP assay using antibody against acetylated histone H3. Immuno-precipitation was performed for each clone cultured in the absence of hygromycin B for the indicated numbers of weeks (w). The cross-linked chromatin before immuno-precipitation (input; in), precipitated without (–), and with (+) the antibody were PCR amplified by the primer sets for 5′LTR (LTR), constitutively expressing HPRT, and non-expressing albumin (alb). In the PCR reaction, template of input (in) was 67% of the immuno-precipitated samples.

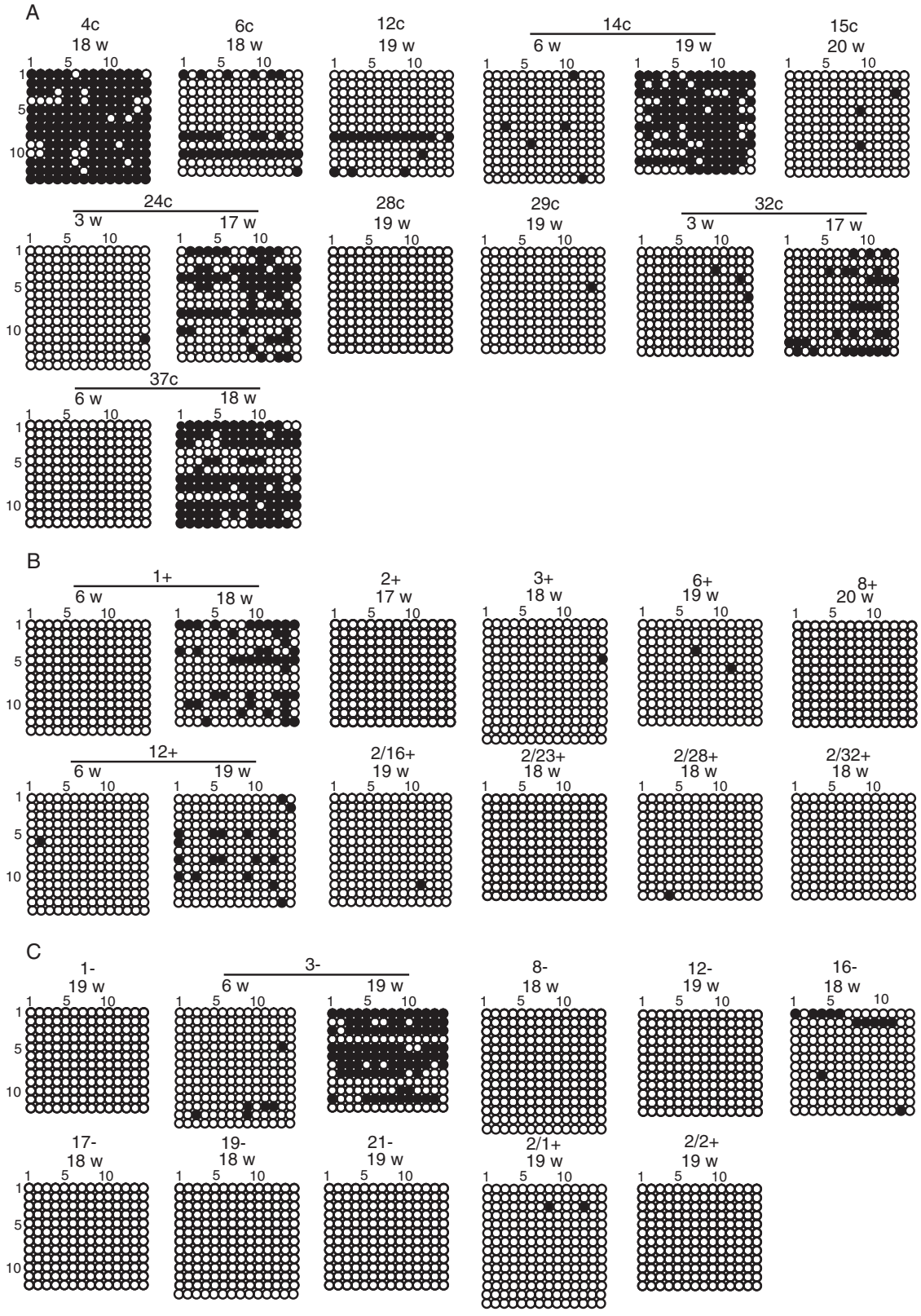

Fig. 5. **DNA methylation level of the 5′LTR region of the isolated clones after long-term culture.** The clones transduced with the reporter gene without Ars insulator (A), inserted with Ars insulator in + (B) and – (C) orientations were subjected to bisulfite modification to analyze the methylation level of the 5′LTR. Before the removal of hygromycin B, one or no CG site was methylated in more than 12 sequences determined in each of the clones. After the indicated numbers of weeks (w), genomic DNA was subjected to bisulfite modification, and then CG methylation was determined. Filled and open circles indicate the methylated and unmethylated CG sites, respectively. Numbers above each panel indicate the 14 positions of CG shown in Fig. 1B. Numbers at the left indicate the numbers of the sequences for which the methylation state was determined.

histone deacetylation, and DNA methylation at the promoter may not be concurrent events.

Of the – clones, the 5′LTR of one clone, 3–, was heavily methylated, but the hrGFP expression and the histone H3 acetylation level in the 5′LTR were maintained. This phenotype apparently is similar to that of 37c, which contained a small population of hrGFP-expressing cells (see Fig. 3, A and C), except for the acetylation level of histone H3. This is another indication that gene silencing, histone deacetylation, and DNA methylation may not occur simultaneously.

DISCUSSION

In the present study, we have shown that the insertion of Ars insulator into the LTRs of retrovirus vector in the – orientation ensured long-term expression of the reporter gene. The Ars insulator inserted in the – orientation was also more effective than that in the + orientation in preventing deacetylation of histone H3 in the 5′LTR (see Table 1). This orientation-dependent anti-silencing effect has also been reported for cHS4 insulator inserted into the LTR of MoMLV vector (*12*). It is likely that the enhancer and/or silencer blocking from the upstream direction may be more important than that from the downstream. Interestingly, Ars insulator inserted in both orientations protected equally against the DNA methylation wave. Although some exceptions exist, we conclude that the Ars insulator ensures long-term expression of the transgene in an orientation-dependent manner by protecting the promoter from histone deacetylation and DNA methylation, acting in a similar manner to the insulator cHS4 found in chicken β-globin gene locus control region (*6*).

Emery *et al.* reported that the activity against position-effect variegation of the cHS4 insulator inserted into MoMLV vector depends on the orientation of the insulator (*12*). Just after the isolation of the clones, the expression level of the reporter gene with the insulator in one of the two orientations is higher than those without the insulator or with the insulator inserted in the other orientation. However, unlike the cHS4 insulator, the expression level of the reporter gene with the Ars insulator showed no significant orientation-dependent difference (data not shown). Using the identical cHS4 insulator and a similar MoMLV vector, Rivella *et al.* reported finding no effect of the insulator on the position-effect variegation at least for the expression level of the reporter gene (*13*). Therefore, use of the expression level of the reporter gene as a marker to evaluate anti-position-effect variegation activity of the insulator should be reconsidered.

Unexpectedly, in half of the clones transduced with the reporter gene without Ars insulator, the hrGFP expression escaped silencing even after long-term culture. This could be explained as follows. The selection of the hrGFP-expressing clones could be biased. Relatively long-term culture of 1–2 wk in the presence of hygromycin B resulted in selection of cells in which the hrGFP gene was integrated into the constitutively active genomic region. In this region, the hrGFP gene and hygromycin resistant gene are likely to be always active.

When the transgene is silenced, it has been reported that an initial step in the silencing is the deacetylation of histones at the promoter. This is followed by the methylation of K9 or K27 of histone H3, and conversion of the region into heterochromatin by recruiting proteins such as HP1 and/or polycomb group proteins (*23*). DNA methylation is proposed to maintain the region in the heterochromatin state thereafter (*23*, *24*). A typical example of silencing of the hrGFP gene is clone 14c, which was completely silenced after long-term culture. Histone H3 in the 5′LTR in this clone was deacetylated after 23 wk of culture, and the 5′LTR promoter was almost completely methylated (Figs. 2–5, panels A). After 6 wk of culture, the hrGFP expression in clone 14c was almost completely silenced. However, the 5′LTR and the histone H3 located in this region remained demethylated and acetylated, respectively (Figs. 2–5 panels A). Complete histone deacetylation occurred only at a later stage, after 23 wk of culture, and the dense DNA methylation in the 5′LTR was observed only after 19 wk of culture. Apparently, the silencing of hrGFP preceded the histone H3 deacetylation. The hrGFP expression seemed to be silenced initially by a mechanism other than histone deacetylation.

This discrepancy in timing of the hrGFP silencing and the histone H3 deacetylation was observed not only for clone 14c but was also for other silenced clones, *i.e.*, 24c, 32c, and 12+. In these clones, histone H3 in the 5′LTR was acetylated even after the silencing of hrGFP expression. The 5′LTR of the reporter gene in clone 24c contained acetylated histone H3 even after 23 wk of culture, despite the complete silencing of hrGFP (Fig. 4A). Similar to this acetylation state, the DNA methylation of this region was also incomplete in clone 24c after 17 wk of culture (Fig. 5A). This result supports the possibility that an unknown silencing mechanism may precede histone deacetylation and be followed by DNA methylation. Such a mechanism for silencing of retroviral vector that precedes DNA methylation is expected in undifferentiated stem cells (*16*). A similar mechanism may exist in C2C12 cells, progenitor cells for skeletal muscle.

In most of the hrGFP-silenced clones, the promoters were heavily methylated after long-term culture. This processive DNA methylation of the 5′LTR was prominent for the reporter gene without Ars insulator (Fig. 5A). However, in two silenced clones transduced with Ars insulator in the + orientation (1+ and 12+), the DNA methylation of the 5′LTR was rather sparse even when the histone deacetylation was developed (Fig. 5B). This discrepancy between the deacetylation of histone H3 and the DNA methylation levels suggests that Ars insulator prevented or delayed the wave of DNA methylation independently of histone deacetylation and/or transgene silencing.

Because of the technical limitation that the 5′LTR in vector plasmid is displaced by the 3′LTR when it integrates into the genome, we could not insert the Ars insulator to surround the reporter gene in different orientations. If we were able to surround the transgene in the orientation to block the enhancer effect both from the 5′ and 3′ directions, the transgene could be expressed in a more stable manner without silencing.

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