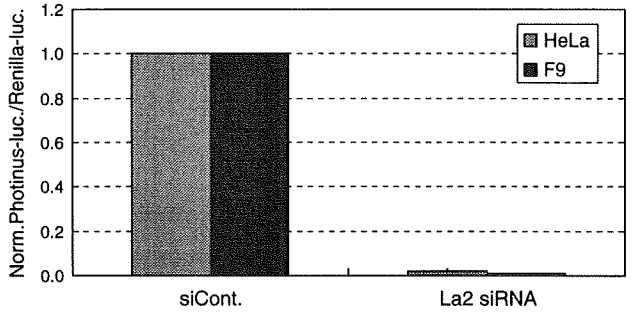

Fig. 6 RNAi activities induced by synthetic siRNA duplex in F9 cells. Chemically synthesized La2 siRNA duplex against the *Photinus luciferase* gene [35] together with pGL3-control (Promega) and phRL-TK plasmids (Promega) carrying *Photinus* and *Renilla luciferase* reporter genes, respectively, were cotransfected into F9 and HeLa (control) cells. Twenty-four hours after transfection, cell lysate was prepared, and dual-luciferase assay was carried out and analyzed as described previously [35]. Data are averages of at least three independent experiments. Error bars represent standard deviations

by using an exogenous (chemically synthesized) siRNA duplex triggering strong RNAi. The La2 siRNA duplex, which can induce strong RNAi activity against the *Photinus luciferase* gene [35], was cotransfected with the target reporter gene into F9 cells. As shown in Fig. 6, consistent with the previous results [35, 36], the cells exhibited potent RNAi, suggesting that F9 cell possesses a full-RNAi machinery. This has been also supported by experiments with a synthetic pre-*let-7* precursor: the expression level of the target reporter gene carrying either PMTS or 3 × BBS was decreased in the presence of the pre-*let-7* precursor (Fig. 7b). Taken together, the data presented here suggest that the expression level of *let-7* greatly influences the level of gene silencing against its target genes.

Effect of transcriptional activity of target gene on gene silencing

In this study, we also examined the effect of transcriptional activity of the target reporter genes on gene silencing. The SV40 and TK promoters, which drive the reporter genes (Fig. 1), appear to have different promoter activities: the transcriptional activity of the SV40 promoter appears to be stronger than that of the TK promoter in the cells examined here (Fig. 8). When suppression level was examined between the promoters, little or no difference in suppression of the reporter gene carrying PMTS was detected, and in contrast, the reporter gene carrying 3 × BBS appeared to exhibit difference in suppression level between the promoters. Table 3 shows relative expression level of the target reporter gene carrying 3 × BBS, when the expression level of the target gene carrying PMTS is given as 1; and the data indicate differences in the relative expression levels between the promoters. Taken together, it is suggested that

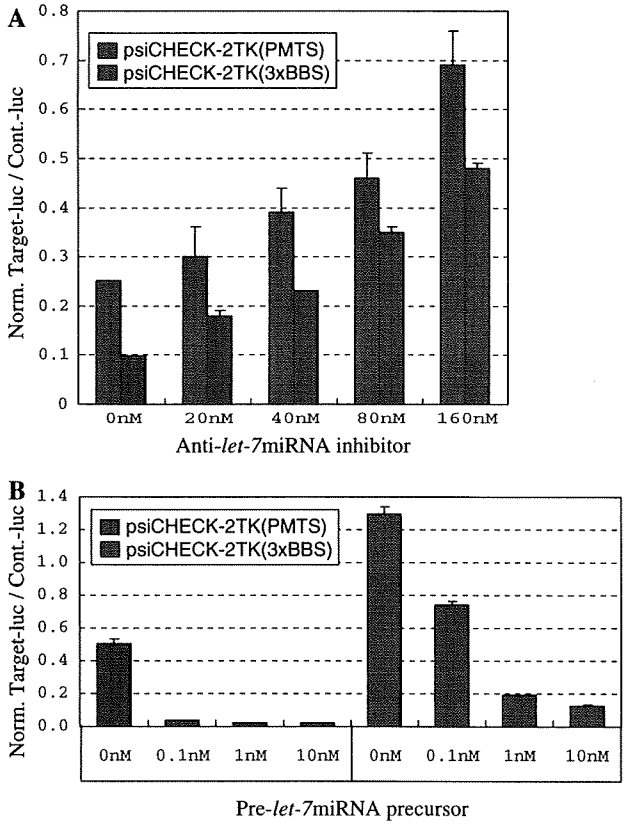

Fig. 7 Dose-dependent suppression of target reporter genes. The psiCHECK-2-TK(PMTS) and psiCHECK-2-TK(3 × BBS) plasmids were cotransfected with an increasing amount of the Anti-miR miRNA inhibitor of *let-7a* (Ambion), from 0 to 160 nM, into 293 cells (**a**), or an increasing amount of the Pre-miR miRNA precursor of *let-7a* (Ambion), from 0 to 20 nM, into F9 cells (**b**). 48 and 24 h after transfection with the inhibitor and precursor, respectively, cell extract was prepared and dual-luciferase assay was carried out. Data are presented as normalized ratios of target (*Renilla*) luciferase activity to control (*Photinus*) luciferase activity as in Fig. 2

different expression levels of one particular target gene undergoing gene silencing by translation inhibition may be reflected in a variety of its gene expression, whereas those undergoing gene silencing by RNAi may be not.

Variety of gene silencing

The data presented here suggest possible parameters conferring a variety of gene silencing: (i) level of endogenous miRNA and (ii) level of target mRNA. In addition, it has been known that various base-pairing interactions can be formed between miRNAs and their target mRNAs [31, 37]. It is conceivable that these parameters presumably raise a great deal of combination between miRNAs and target mRNAs in a qualitative and quantitative manner. Accordingly, the resultant gene silencing based on such a combination may yield various levels of suppression against target mRNAs, thereby conferring a variety of gene expression.

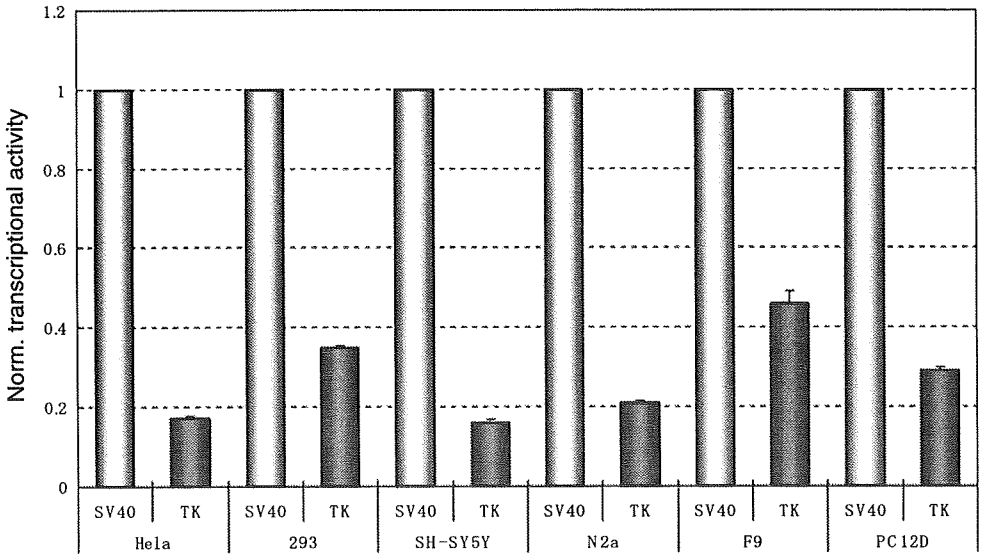

Fig. 8 Transcriptional activity. The transcriptional activities of the SV40 and TK promoters which drive the target reporter (*Renilla luciferase*) gene were investigated in the cells (indicated). The psiCHECK-2 and psiCHECK-2-TK empty plasmids were introduced into the cells and the expressed luciferases were examined as in Fig. 2. Ratios of normalized *Renilla* luciferase activity to control *Photinus* luciferase activity are shown: the ratio of the *Renilla* luciferase activity in the presence of psiCHECK-2-TK is normalized to the ratio obtained in the presence of psiCHECK-2 in each cell. Data are averages of at least three independent experiments. Error bars represent standard deviations

Table 3 Relative expression levels of target reporter gene carrying 3 × BBS

Cell name	SV40[a]	TK[a]
HeLa	9.09	2.85
293	4.84	2.53
SH-SY5Y	5.12	2.57
N2a	21.7	16.3
F9	3.76	4.44
PC12D	11.4	6.1

The relative expression level is indicated when the expression level of the target reporter gene carrying PMTS is given as 1 under each promoter

[a] Used promoters for driving the target reporter genes

Acknowledgements We would like to thank T. Fukushima (Mitsubishi Rayon) for his helpful assistance. This work was supported in part by research grants from the Ministry of Health, Labour and Welfare of Japan and by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

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