Enhanced Gene Silencing in Cells Cured of Persistent Virus Infection by RNA Interference[▽]

Isabelle Pelletier,^{1,2} Aure Saulnier,^{1†} Cynthia Brisac,^{1,2} Sophie Jegouic,^{1,2} Nicolas Vabret,³ Frédéric Tangy,³ Bruno Blondel,^{1,2} and Florence Colbère-Garapin^{1,2*}

Institut Pasteur, Unité de Biologie des virus entériques, 25 rue du Dr Roux, 75015 Paris, France¹; Institut National de la Santé et de la Recherche Médicale U994, Paris, France²; and Institut Pasteur, Unité de Génomique virale et vaccination, 25 rue du Dr Roux, 75015 Paris, France³

Received 30 September 2009/Accepted 5 April 2010

We compared HEP-2-derived cells cured of persistent poliovirus infection by RNA interference (RNAi) with parental cells, to investigate possible changes in the efficiency of RNAi. Lower levels of poliovirus replication were observed in cured cells, possibly facilitating virus silencing by antiviral small interfering RNAs (siRNAs). However, green fluorescent protein (GFP) produced from a measles virus vector and also GFP and luciferase produced from plasmids that do not replicate in human cells were more effectively silenced by specific siRNAs in cured than in control cells. Thus, cells displaying enhanced silencing were selected during curing by RNAi. Our results strongly suggest that the RNAi machinery of cured cells is more efficient than that of parental cells.

Small interfering RNAs (siRNAs) mediate RNA interference (RNAi), a natural biological phenomenon regulating a wide range of cellular pathways (8, 20). RNAi-based therapies with siRNAs or small hairpin RNAs (shRNAs) have been developed against several viral infections, and a reduction of the viral yield by several orders of magnitude has frequently been obtained (4, 9). However, virus clearance from cells and the complete cure of persistent virus infections have only rarely been reported (24, 25). We have developed several models of persistent virus infection by using poliovirus (PV), a positive-strand RNA virus of the *Picornaviridae* family (5, 7, 16, 21). We previously studied the effects of antiviral siRNAs applied months after the infection of HEP-2 cells with a persistent PV mutant (7, 25). We used a mixture (“the Mix”) of two synthetic siRNAs targeting the viral RNA genome in the 5′ noncoding (NC) region and the 3D polymerase (3D^{pol}) (siRNA-5′NC and siRNA-3D^{pol}, respectively; synthesized by Sigma-Proligo). When repeated transfections with the Mix were performed in persistently PV-infected cultures, most cultures stopped producing virus (25). Here, we investigate the important issue of changes in RNAi efficacy following siRNA treatment, 2 to 5 months after the cure. The efficiency of gene silencing in cells was stable during this period.

We used the HEP-Q4 and -Q5 cell lines, which were cured of persistent PV infection after transfections with the Mix (25). The cured cells and their parental cell line, HEP-2, had similar growth rates (data not shown). To compare PV silencing efficiencies in the three cell lines, they were trans-

fected either with the Mix or with an irrelevant siRNA (siRNA-IRR) in the presence of Lipofectamine 2000 (Invitrogen) in 24-well plates as previously described (25). Treated and mock-treated cells were infected 16 h posttransfection with PV strain Sabin 3, at a multiplicity of infection (MOI) of 1 50% infectious dose (ID₅₀) per cell. The viral progeny was titrated 24 h postinfection, as previously described (16). HEP-Q4 and HEP-Q5 were permissive to PV infection, although viral yields were about 1 log lower in these cells than in HEP-2 cells (Fig. 1A). Virus silencing was observed in all three cell lines treated with the Mix; however, silencing was significantly more efficient in HEP-Q4 (≈2.2 times more efficient; *P* = 0.013, Student's *t* test) and HEP-Q5 (≈5.6 times more efficient; *P* = 0.015) than in HEP-2 cells (Fig. 1A and B). Similar results were obtained with an shRNA (Thermo Scientific) targeting the same region as the siRNA-5′NC (data not shown).

We investigated whether the differences in silencing efficiencies between the three cell lines were due to differences in siRNA transfection efficiency by transfecting HEP-2, HEP-Q4, and HEP-Q5 cells with fluorescein isothiocyanate-conjugated siRNA (siRNA-FITC; 20 pmol/well; Cell Signaling) and testing them between 4 and 48 h posttransfection. The fluorescence of transfected cells was measured with a FACScan flow cytometer (Becton Dickinson), and data were analyzed with CellQuest software (Becton Dickinson). The percentages of siRNA-FITC-positive cells were similar for all cell types (Fig. 2A). The mean fluorescence per positive cell and the percentage of cells displaying fluorescence peaked 16 and 24 h posttransfection, respectively, and decreased thereafter (Fig. 2). These findings suggest both that the presence of siRNAs in cells was similarly transient in the three cell types, as previously reported (27), and that the high silencing efficiencies in cured cells were not a consequence of higher transfection efficiencies. All subsequent experiments were performed between 16 and 40 h posttransfection.

Fluorescence-activated cell sorting (FACS) plots for gran-

* Corresponding author. Mailing address: Institut Pasteur, Unité de Biologie des virus entériques, 25 rue du Dr Roux, 75015 Paris, France. Phone: 33 1 45 68 87 64. Fax: 33 1 40 61 33 67. E-mail: florence.colbere-garapin@pasteur.fr.

† Present address: Agence Française de Sécurité Sanitaire des Aliments, BP 53, 22440 Ploufragan, France.

[▽] Published ahead of print on 14 April 2010.

FIG. 1. Efficiency of enterovirus silencing in HEp-2, HEp-Q4, and HEp-Q5 cells after transfection with specific siRNAs. (A) Yield of progeny virus produced by cells infected at an MOI of 1 ID₅₀, 16 h posttransfection with the antiviral Mix containing two anti-PV siRNAs (20 pmol), the irrelevant siRNA-IRR (20 pmol), or no siRNA. Samples were harvested 24 h postinfection. Each bar represents the mean value $\pm$ SEM of six infected cultures from three independent experiments. (B to E) For each cell line, silencing efficiency is expressed as the ratio of infectious virus yield (titer in ID₅₀/ml) in the presence of the irrelevant siRNA-IRR to infectious virus yield (titer in ID₅₀/ml) in the presence of the antiviral siRNAs in cured cells, normalized with respect to the silencing efficiency in HEp-2 cells. S2, PV strain Sabin 2. (F) GFP silencing efficiency for each cell line is expressed as a ratio $[1 - (\text{mean GFP levels in the presence of siRNA-eGFP})/(\text{mean GFP levels in the presence of siRNA-IRR})]$ in cured cells, normalized with respect to the efficiency of silencing in HEp-2 cells. Each bar represents the mean value $\pm$ SEM of at least four cultures from two independent experiments. *, $P < 0.05$ based on Student's t test comparing HEp-Q4 and HEp-Q5 with HEp-2 cells.

ularity versus cell size were very similar for the three cell lines (Fig. 2C), as were those for cell numbers versus fluorescence (not shown), suggesting highly related cell populations. Although highly probable, it remains to be confirmed that the cured cells originated from a subpopulation of HEp-2 cells.

Virus silencing was also investigated in cured cells infected with Sabin 2 or coxsackievirus A17 (CAV17) strain 67591 (22) or in cells transfected with Sabin 2 RNA. The experimental conditions used for Sabin 2 and CAV17 were identical to those

for Sabin 3, except that only the 3D polymerase was targeted by siRNAs. Sabin 2 RNA (1 μ g) was prepared as previously described (12) and used with siRNA-3D^{pol} (20 pmol/well) for the cotransfection of cells in the presence of Lipofectamine 2000. Virus yields were determined 7.5 h after transfection. In all cases, virus silencing was more effective in HEp-Q4 and -Q5 cells than in HEp-2 cells (Fig. 1C to E). Additional experiments were performed with a PV replicon encoding the green fluorescent protein (GFP), PV-eGFP (28) (2 μ g/well), which was used with siRNA-eGFP (20 pmol/well; Ambion) for cotransfection. GFP fluorescence was measured by flow cytometry, 16 h after transfection. As for PV, a higher silencing efficiency was observed in cured cells than in HEp-2 cells (Fig. 1F).

We then investigated whether the lower level of viral multiplication in HEp-Q4 and -Q5 cells in the absence of siRNAs involved an entry or postentry step. We quantified the expression of the PV receptor (CD155) at the surface of cells. We used flow cytometry after indirect immunofluorescence labeling with anti-CD155 antibodies, as previously described (16). More than $98.4\% \pm 2\%$ (mean $\pm$ standard error of the mean [SEM]) of cured cells, like HEp-2 cells, tested positive for CD155 (data not shown). In the absence of siRNAs, a decrease in viral replication was also observed in HEp-Q4 and -Q5 cells infected with the Sabin 2 PV strain in cells, in which the early stages of the viral cycle were bypassed by transfection with Sabin 2 RNA, and in cells infected with the CAV17 virus, which uses a cell receptor other than CD155 (12) (data not shown). Together, these results suggest that PV multiplication is reduced at a postentry step, probably at replication, in cured cells.

We investigated whether PV silencing was also enhanced in other HEp-derived cells in which Sabin 3 PV multiplication was reduced by using HEp-S31 (cl18) cells that had been cured of persistent PV infection by growth at a supraoptimal temperature rather than by RNAi (2). PV yield was ≈ 1.6 logs lower in HEp-S31 (cl18) cells than in HEp-2 cells (data not shown). Sabin 3 PV silencing in HEp-S31 (cl18) cells was 1.7 ± 0.9 times more effective (mean of six experiments) than that in HEp-2 cells (relative efficacy of 1) (data not shown), but this difference was not significant. However, these results do not exclude the possibility that reduced PV replication facilitates PV silencing by the Mix in cured cells. We therefore pursued our work with a different virus.

We investigated whether the high silencing efficiency in HEp-Q4 and -Q5 cells was specific to enteroviruses by using a measles virus expressing GFP, MV-eGFP (26), and siRNA-eGFP to silence GFP expression. Cells were transfected with either siRNA-eGFP or siRNA-IRR, infected with MV-eGFP (1 ID₅₀ per cell, 16 h posttransfection), and the GFP silencing efficiency was determined 40 h posttransfection by flow cytometry. For each cell line, silencing efficiency was expressed as a percentage $\{[1 - (\text{percentage of siRNA-eGFP-transfected cells expressing GFP})/(\text{percentage of siRNA-IRR-transfected cells expressing GFP})] \times 100\}$. GFP silencing was significantly stronger in HEp-Q4 cells ($\approx 14\%$; $P = 0.048$) and HEp-Q5 cells ($\approx 17\%$; $P = 0.010$) than in HEp-2 cells (Fig. 3A). There was no significant difference in the silencing efficiency of GFP between HEp-Q4 and -Q5 cells (Fig. 3A). The anti-PV Mix did not

FIG. 2. Transfection efficiencies of fluorescein-conjugated siRNAs in HEP-2, HEP-Q4, and HEP-Q5 cells. A fluorescein siRNA-FITC (20 pmol) was used to transfect each of the three cell lines in the presence of Lipofectamine 2000. Fluorescent cells were analyzed 4 to 48 h posttransfection by using a FACScan flow cytometer (Becton Dickinson). The percentage of fluorescent cells (A) and the mean fluorescence per positive cell, in arbitrary units (B), are shown. Each bar represents the mean value $\pm$ SEM. (C) Representative FACS plots (cell granularity versus cell size), showing the similarities between the three cell populations.

silence GFP expression (data not shown), indicating that the silencing of GFP was not due to anti-PV siRNAs persisting in cured cells months after the initial treatment.

To test whether the high silencing efficiency in HEP-Q4 and -Q5 cells was dependent on viral infection, plasmid vectors pEGFP-C1 (Clontech Laboratories) and pRL-CMV (Promega) were used to generate GFP (6) and *Renilla* luciferase (18), respectively. These plasmids do not replicate in human cells. Cells (10^6) were cotransfected with pEGFP-C1 (1 μ g) and siRNAs (20 pmol) in the presence of Lipofectamine 2000, as recommended by the manufacturer. GFP fluorescence was analyzed by flow cytometry 40 h posttransfection. Silencing efficiencies were expressed as a percentage $\{[1 - (\text{mean GFP levels in the presence of siRNA-eGFP})/(\text{mean GFP levels in the presence of siRNA-IRR})] \times 100\}$. Mean silencing efficiency was significantly higher in HEP-Q4 ($\approx 15\%$; $P = 0.003$) and HEP-Q5 ($\approx 15\%$; $P = 0.002$) cells than in HEP-2 cells (Fig. 3B). The efficiency with which the GFP encoded by pEGFP-C1 was silenced was similar in HEP-Q4 and -Q5 cells.

The efficacy of siRNAs was then assessed with pRL-CMV, which encodes the *Renilla* luciferase and Silencer *Renilla* luciferase (AM4630; Ambion). Cells (10^6) were cotransfected with the plasmid (100 ng) and either specific or irrelevant siRNA (7 pmol) in the presence of Lipofectamine

2000. Luciferase assays were performed with a Dual-Glo luciferase assay system (Promega), as recommended by the manufacturer at 40 h posttransfection, and luminescence was measured with a luminometer (Centro LB960; Berthold). The results of the sensitive luciferase assays confirmed that the relative efficiency of silencing was significantly higher in cured than in parental cells (Fig. 3C). By contrast, results obtained in HEP-S31 (cl18) cells, cured without siRNAs, were not significantly different from those obtained in control HEP-2 cells (data not shown), strongly suggesting that the treatment of HEP-Q4 and -Q5 cells with specific siRNAs selected cells in which siRNAs mediated silencing more efficiently than in parental cells.

The difference in silencing efficiency between cured and HEP-2 cells may be due to differences in the abundance and/or efficacy of cellular factors involved in gene silencing. Some major actors of the RNAi pathway, particularly those associated with the RNA-induced silencing complex (RISC), have been identified (3, 10, 13, 19). The active endonucleolytic core of the RISC includes the guide strand of the siRNA and a slicer protein called Argonaute 2 (Ago2) (17). We used Western blotting to study Ago-2 and other factors contributing to the function of RISC (3, 10, 11, 14, 19, 23): the endonuclease Dicer, the transactivation response RNA binding protein (TRBP), the protein activator of double-stranded

FIG. 3. Efficiency of GFP and luciferase silencing in Hep-2, Hep-Q4, and Hep-Q5 cells after transfection with specific siRNAs. (A and B) GFP silencing, expressed as a percentage calculated for each cell line as follows: $\{[1 - (\text{GFP expression in the presence of siRNA-eGFP})/(\text{GFP expression in the presence of the irrelevant siRNA-IRR})] \times 100\}$. (A) Cells were infected 16 h posttransfection with a measles virus encoding eGFP (MV-eGFP [26]) at an MOI of 1 ID₅₀/cell, and fluorescent cells were analyzed 24 h after infection (40 h posttransfection). Each bar represents the mean value $\pm$ SEM of three independent experiments. (B) Cells were cotransfected with pEGFP-C1 and siRNA-eGFP or siRNA-IRR and analyzed 40 h later. Each bar represents the mean value $\pm$ SEM of four independent experiments. (C) Luciferase silencing efficiency for each cell line, expressed as the ratio

RNA-dependent protein kinase (PACT), and the RNA helicase A (RHA) (Fig. 4). Exportin 5, which plays a role upstream from the dicing process in the export of small RNA precursors (29), was included as a control.

Proteins (30 to 50 μ g) from each cell line were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10 to 20% Tricine gels; Invitrogen) and transferred to nitrocellulose membranes (Amersham Biosciences) as previously described (1). The membranes were incubated with one of the following primary antibodies (1): anti-Ago2 monoclonal antibody (MAb; Abcam), anti-RHA MAb (Abcam), and anti-TRBP2 MAb (Santa Cruz Biotechnology); rabbit antibodies against Dicer (Santa Cruz Biotechnology); anti-PACT MAb (Santa Cruz Biotechnology), and anti-Exportin 5 MAb (Abcam). The antiactin MAb (AC-40; Sigma-Aldrich) was used to check for equal protein loading. Membranes were then washed and treated with appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) for 2 h at room temperature. Protein bands were detected with an enhanced chemiluminescence detection kit (ECL⁺; Amersham Biosciences) and a G:box (Syngene).

Exportin 5, Dicer, Ago-2, and RHA were similarly abundant in all three cell lines (Fig. 4A to D), suggesting that quantitative differences in protein levels were unlikely to be responsible for the enhanced silencing in Hep-Q4 and -Q5 cells. There was significantly more TRBP in Hep-Q4 ($\approx 21\%$; $P = 0.026$) and Hep-Q5 ($\approx 28\%$; $P = 0.016$) cells than in Hep-2 cells, as indicated by the results of nine experiments (Fig. 4E and F). The specificity of the anti-TRBP antibody was checked on extracts of Hep-2 cells transfected with a plasmid encoding TRBP, pcDNA-TRBP (14), with and without silencing by siRNA-TRBP (Fig. 4G). GFP silencing was not enhanced in Hep-2 cells overproducing TRBP, and it was not decreased by downregulating TRBP gene expression with siRNA-TRBP (data not shown). These results suggest that the high levels of TRBP in the cured cell lines are not the cause of the enhanced silencing in these cells.

There was less TRBP protein in Hep-S31 (cl18) cells (2) than in Hep-2 and other control cells (IMR5) (Fig. 4H), indicating that high levels of TRBP are not necessarily selected in cells persistently infected with PV. PACT was slightly downregulated in the cured cells (Fig. 4I). Moreover, PACT is unlikely to be involved in the enhanced silencing in cured cells, because we used synthetic siRNAs and PACT functions principally during siRNA production by Dicer (14). We did not investigate the activities or subcellular distributions of the various factors involved in RNAi in the three cell lines, and they may differ. It is also possible that other factors, not tested here, contribute to the efficacy of siRNAs in cured cells. The molec-

of luciferase activity in the presence of the irrelevant siRNA-IRR to luciferase activity in the presence of the specific siRNAs in cured cells, normalized with respect to silencing efficiency in Hep-2 cells. Relative efficiencies are shown as in Fig. 1 for luciferase, because the enzymatic reaction amplified the signal. Each bar represents the mean value $\pm$ SEM of triplicates from three independent experiments. *, $P < 0.05$ based on Student's *t* test comparing Hep-Q4 and Hep-Q5 with Hep-2 cells.

FIG. 4. Comparative analysis of proteins involved in RNAi in HEP-2, HEP-Q4, and HEP-Q5 cell lines. Whole-cell lysates were tested for Exportin 5 (A), Dicer (B), Ago-2 (C), the helicase RHA (D), TRBP (E to H) and PACT (I) by Western blotting with the corresponding specific antibodies. Blots were subsequently stripped and reprobed with antiactin antibodies to confirm equal protein loading. (E and F) TRBP levels in HEP-Q4 and HEP-Q5 cells were determined by densitometry and are plotted in arbitrary units, as ratios relative to the level of actin and to the level of TRBP in HEP-2 cells. In panel F the symbols correspond to TRBP levels determined in nine different experiments. (G) TRBP levels in HEP-2 cells transfected with pcDNA-TRBP (14) and in cells cotransfected with pcDNA-TRBP and siRNA-TRBP. (H) TRBP levels were compared in human IMR5 cells, HEP-S31 (cl18) cells previously cured of persistent PV infection by growth at a supraoptimal temperature, and the control HEP-2 cell line. TRBP/actin densitometry and PACT/actin densitometry results are indicated in arbitrary units in the histograms below the corresponding Western blot results shown in panels H and I.

ular details of the mechanism involved remain to be determined.

Overall, our results suggest that both a decrease in viral replication and the enhancement of gene silencing contributed to the mechanism by which cells persistently infected with poliovirus were cured by RNAi. Our results also indicate that cells displaying enhanced silencing may be selected during treatment with siRNAs. This may result in profound changes to cell phenotype, because RNAi plays an essential role in the regulation of cellular gene expression (15).

We thank Dong-Yan Jin and Addgene for providing pcDNA-TRBP and Nicolas Escriou and Marco Vignuzzi for providing the PV-GFP replicon. We thank Carla Saleh, Pierre-Olivier Vidalain, Arnaud Autret, Maël Bessaud, Marie-Line Joffret, and Francis Delpeyroux for helpful discussions.

This work was supported by grants from the Institut Pasteur (Transverse research program PTR 276), the Agence Nationale de la Recherche (ANR-09-MIEN-019), and the Fondation pour la Recherche Médicale (DMI20091117313). A.S. was supported by grants from the Ministère de l'Éducation Nationale, de la Recherche et de la Technologie, and S.J. was supported by grants from the Fondation Mérieux and Rotary International.

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