Sensitive assay of RNA interference in *Drosophila* and Chinese hamster cultured cells using firefly luciferase gene as target

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Received 18 April 2000; revised 19 July 2000; accepted 21 July 2000

Edited by Takashi Gojobori

Abstract A sensitive cellular assay system for RNA interference was developed using the firefly luciferase gene as target. RNA interference was noted not only in *Drosophila* cultured cells but Chinese hamster cells (CHO-K1) as well, although double-stranded RNA required for the latter was 2500 times more than for the former. Cognate double-stranded RNA as short as 38 bp was found to be still capable of inducing RNA interference in *Drosophila* cultured cells.

Key words: RNA interference; Double-stranded RNA; Chinese hamster; Cultured cell; *Drosophila*

1. Introduction

RNA interference (RNAi) is a new technique for post-transcriptional gene silencing (PTGS), in which target gene activity is specifically abolished with cognate double-stranded RNA (dsRNA; reviewed in [1–3]). RNAi resembles in many aspects PTGS in plants [4,5] and has been detected in many invertebrates including trypanosome [6], hydra [7], planaria [8], nematode [9,10] and fruit fly (*Drosophila melanogaster*; [11–13]). It may be involved in the modulation of transposable element mobilization and antiviral state formation [1–3].

In contrast to invertebrates, RNAi has been applied only with limited success to vertebrates. In zebrafish, 20–30% of the activity of three genetically characterized genes was abolished in a partially sequence-specific manner by dsRNA injected into eggs [14]. DsRNA injected into mouse pre-implantation embryos and oocytes eliminated some target gene activity, while little or no RNAi activity could be detected in embryos at 6.5 days post-implantation [15]. Vertebrate cells would thus appear potentially susceptible to RNAi but RNAi activity may be frequently masked by unknown mechanisms.

Although the precise molecular mechanism of RNAi remains to be clarified, recent experiments show that, at least in *Drosophila*, RNAi includes the formation of protein–RNA complexes associated with sequence-specific RNase activity [16–18]. Cell-free extracts of *Drosophila* embryos may possess activity for assembling RNAi enzyme complexes [16,18]. DsRNA, broken into pieces and incorporated into a complex, may serve as a target gene determinant.

Here, we developed a sensitive cellular system of RNAi using the firefly luciferase gene as target and showed that RNAi occurs not only in *Drosophila* cultured cells but also in Chinese hamster cells (CHO-K1 cells), although dsRNA required for the latter was much more than for the former.

2. Materials and methods

2.1. Cell culture

Drosophila S2 cells and Chinese hamster CHO-K1 cells (RIKEN Cell Bank), respectively, were cultured in Schneider medium (Gibco BRL) at 25°C and in Dulbecco's modified Eagle's medium (Gibco BRL) at 37°C. Both media were supplemented with 10% heat-inactivated fetal bovine serum (Mitsubishi Kasei) and antibiotics (10 units/ml of penicillin (Meiji) and 50 μg/ml of streptomycin (Meiji)).

2.2. DsRNA preparation

pGL3-Control (Promega) and pRL-TK (Promega), respectively, are expression plasmids of *Photinus pyralis* (firefly) *luciferase* (*luc*) gene ([19]; accession number, U47296) and *Renilla reniformis* (sea pansy) *luc* (accession number, A28028). In pGL3-Control, *luc* was terminated with SV40 promoter and polyadenylation signal, and hence, 1.9-kb long RNA is expected to be produced in transfected *Drosophila* or Chinese hamster cells (see Fig. 1). In pRL-TK, *luc* was flanked with HSV-TK promoter and SV40 polyadenylation signal. In almost all experiments, *P. pyralis luc* was used as target gene for RNAi while *R. reniformis luc* served as a reference and was used for correction of transfection variation. DsRNAs were prepared using *P. pyralis*, *Photuris pennsylvanica* (firefly; S.Z., unpublished; accession number, D12415) or *R. reniformis luc* cDNA or *D. melanogaster frizzled 2* cDNA (*Dfz2*; [20]) as templates (see Fig. 1). DNA fragments with various lengths were cloned into pBluescript carrying T7 and T3 promoters (Stratagene). Sense-strand (ss)RNA and antisense-strand (as)RNA were synthesized using PCR-amplified template DNA and RiboProbe in vitro transcription systems (Promega) with T7 or T3 RNA polymerase. RNA was capped by the addition of $m^7G(5')ppp(5')G$ cap analog (Ambion) to the reaction mixture. DsRNA was prepared by annealing ssRNA and asRNA in 10 mM Tris–HCl (pH 7.5) with 20 mM NaCl. The reaction mixture was heated at 95°C for 10 min, then gradually cooled down to room temperature, and incubated for 16–20 h at room temperature. Formation of dsRNA was monitored by electrophoresis on a 2% agarose gel in TBE buffer. Under the present condition, most, if not all, single-stranded RNA was converted to dsRNA.

2.3. Construction of expression plasmid encoding a fold-back type of RNA

Expression plasmid encoding an inverted repeat of a portion of the *P. pyralis luc* gene was constructed using a derivative of pSC, a vector including paired *Sfi*I and *Cpo*I restriction sites (S.Z., unpublished). Two 696-bp long, *P. pyralis luc* gene fragments, almost identical in sequence to each other, were prepared by PCR amplification and introduced into *Sfi*I and *Cpo*I sites of the pSC derivative in an oppo-

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Abbreviations: RNAi, RNA interference; dsRNA, double-stranded RNA; *luc, luciferase*; *Dfz2*, *Drosophila melanogaster frizzled 2*; PKR, dsRNA-responsive protein kinase

0014-5793/00/$20.00
PII: S0014-5793(00)01883-4

site orientation to generate pSC-PL696 (see Fig. 1). CHO cells transfected with pSC-PL696 are expected to produce only fold-back RNA in which complementary target gene sequences form a double helix.

2.4. Transfection and RNAi activity assay

S2 and CHO-K1 cells, respectively, were inoculated at 1×10^6 and 3×10^5 cells/ml in each well of 24-well plate. After 1 day, using the calcium phosphate precipitation method, cells were transfected with either a mixture consisting of *P. pyralis luc* dsRNA (80 pg–3 μg), pGL3-Control DNA (2.6 μg) and pRL-TK DNA (0.05 or 0.5 μg) or that including *R. reniformis luc* dsRNA (0.001–2 μg), pRL-TK (2 μg) and pGL3-Control (1 μg). Cells were harvested 20 h after transfection and luciferase activity was measured using Dual-Luciferase reporter assay system (Promega). *R. reniformis* luciferase is unrelated in sequence and substrate specificity to firefly luciferases used here, and served as an internal control in the *P. pyralis luc* RNAi assay; *P. pyralis luc* was used as an internal control on assaying *R. reniformis* RNAi activity. The activity of *R. reniformis* and *P. pyralis luc* genes was measured according to the manufacturer's instruction.

3. Results and discussion

3.1. RNAi in Drosophila cultured cells

Firefly luciferase serves as a highly sensitive assay for gene expression in vertebrate and invertebrate cultured cells [21]. *P. pyralis luc* was used as target gene and dsRNA was examined for capacity to reduce the activity of this gene expressed transiently in *Drosophila* S2 cells (this section) and mammalian cells (Section 3.2). Transfection variation was normalized using the activity of co-transfected *R. reniformis luc*. The size and location of dsRNA used are indicated in Fig. 1.

RNAi was recently shown to occur in *Drosophila* cultured cells [11], but no systematic quantitative analysis has been reported to date. Examination was thus initially made to determine how RNAi effects in *Drosophila* S2 cells vary depending on sequence, length or quantity of dsRNA used (Fig. 2A). As anticipated, little or no reduction in *P. pyralis* luciferase activity could be detected on using 6 pg–1 μg per dish of dsRNA homologous to *Dfz2*, a *Drosophila* gene unrelated in sequence to *P. pyralis luc*. *P. pyralis* luciferase activity, however, was significantly reduced in the presence of cognate dsRNA ranging in molecular size from 38 to 1662 bp, although the amount of dsRNA required for effective RNAi varied as a function of dsRNA length. Cognate dsRNAs with more than 356 bp exhibited maximum levels of RD_{50} (50% reduction in *P. pyralis* luciferase activity; Fig. 1), a finding consistent with previous data for other systems [6,16,17]. More than 0.01 μg per dish of 600-bp long cognate dsRNA virtually eliminated *P. pyralis* luciferase activity, indicating that RNAi is capable of abolishing target gene activity almost completely under optimal conditions. Unlike other systems with no or little RNAi activity associated with dsRNA 100 bp in length or less [6,16,17], 38- and 72-bp cognate dsRNAs exhibited apparent RNAi activity in the present system. RNAi activity of the former and latter, respectively, was 1/135 and 1/6 that of 356–1344-bp dsRNAs. The longest prepared dsRNA (1662-bp long dsRNA) was 3–4 times less efficient in RNAi induction than shorter preparations (356–1344-bp dsRNA), possibly, since, for one reason, only 1662-bp dsRNA includes the entire coding sequence of *luc* (see Fig. 1).

RNAi may not necessarily require strict sequence homology between target gene and dsRNA. Thus, dsRNA prepared using *P. pennsylvanica luc*, possessing 67% homology to *P. pyralis luc*, was examined for capacity to reduce *P. pyralis* luciferase activity. As shown in Fig. 2B, no appreciable RNAi

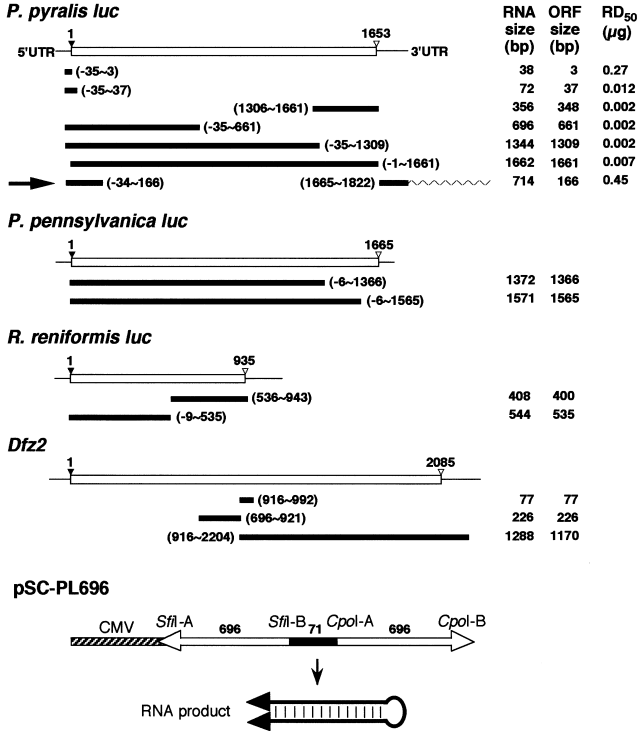

Fig. 1. The size and location of dsRNA are schematically shown. DsRNA was prepared using four genes, *P. pyralis luc*, *P. pennsylvanica luc*, *R. reniformis luc* and *Dfz2*, while fold-back RNA was produced within cells transfected with pSC-PL696. In three *luc* genes and *Dfz2*, open boxes show ORF regions and thin horizontal lines indicate the 5′UTR or 3′UTR sequences, while filled boxes show the size and location of dsRNA. Numerals in parentheses indicate the terminal positions of corresponding dsRNA. In the case of dsRNA prepared from *P. pyralis luc*, RNAi effects shown by RD_{50} are indicated in the right margin. The horizontal thick arrow shows the structure of a 714-bp long composite RNA. This composite dsRNA consists of two target gene-homologous sequences (−34–166, 1655–1822) and a 346-bp long vector sequence labeled with a thin zig-zag line. That is, 368 bp of the composite dsRNA are homologous to the target sequence, while the remaining 346 bp are not. In pSC-PL696, the size and location of *P. pyralis luc* fragments are indicated by open boxes labeled with arrows showing the 5′-3′ orientation of sense RNA. CMV; cytomegalovirus promoter. CHO-K1 cells transfected with pSC-PL696 are capable of producing fold-back RNA in which two complementary *P. pyralis luc* sequences form a double helix.

effect was detected, indicating that effective RNAi requires more than 67% nucleotide sequence homology between target genes and dsRNA. *Caenorhabditis elegans* genes encoding body-wall muscle myosin heavy chains were previously shown weakly inactivated through cross-interference with dsRNA possessing 87% homology [3,9]. Accordingly, the critical cut-off point of cross-interference in RNAi may be between 87 and 67%.

One unexpected finding was that a 368-bp long cognate dsRNA associated with 346-bp long dsRNA unrelated in sequence to a target gene was quite inefficient as an RNAi reagent, being less so than 38-bp dsRNA (Figs. 1 and 2A). DsRNA unrelated in sequence to a target gene may thus serve as an inhibitor of the RNAi activity of covalently bound, cognate dsRNA. According to a recent model of RNAi in *Drosophila* [17,18], dsRNA incorporated into cells is broken into pieces 21–23 bp in length and serves as a target determinant in putative protein–RNA complexes, sequence-specific

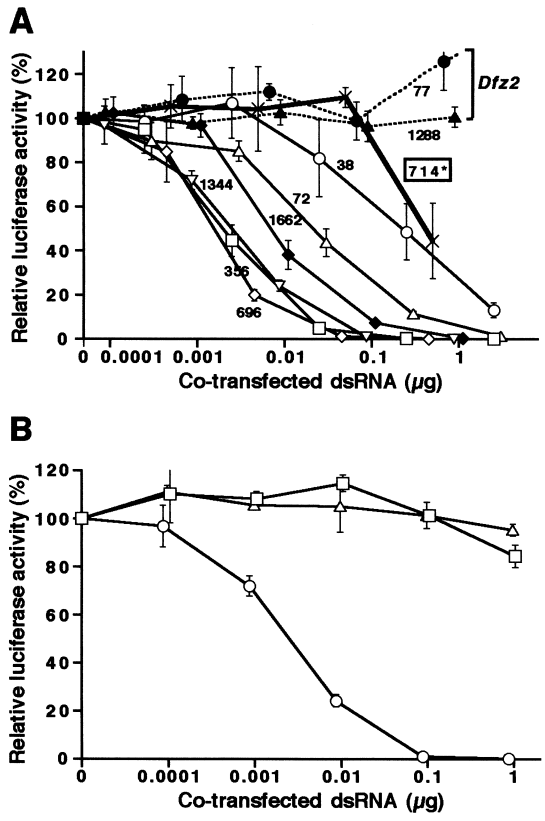

Fig. 2. RNAi effect in *Drosophila* S2 cells. A: Dependency on dsRNA size. S2 cells were transfected with a target gene (2.6 μg of pGL3-Control) and 0.05 μg of pRL-TK with various sizes of *P. pyralis luc* dsRNA. As a control, two *Dfz2* dsRNAs, 77 bp and 1288 bp in length, were used. More than three independent assays were carried out and averaged. Open circles, *P. pyralis* dsRNA 38-bp long. Open triangles, *P. pyralis* dsRNA 72-bp long. Open squares, *P. pyralis* dsRNA 356-bp long. Open diamonds, *P. pyralis* dsRNA 696-bp long. Open inverted triangles, *P. pyralis* dsRNA 1344-bp long. Filled diamonds, *P. pyralis* dsRNA 1662-bp long. Crosses with thick lines and labeled 714* in box, 714-bp long, composite dsRNA with a 368-bp long sequence homologous to *P. pyralis luc* target gene. Closed circles, *Dfz2* dsRNA 77-bp long. Closed triangles, *Dfz2* dsRNA 1288-bp long. *P. pyralis* luciferase activity, normalized using *R. reniformis* luciferase activity, was plotted against the amount of dsRNA used (μg). B: Effects of nucleotide sequence homology on RNAi. RNAi activity of *P. pyralis* dsRNA and *P. pennsylvanica* dsRNA were compared. Open circles, *P. pyralis* dsRNA 1344-bp long. Open triangle, *P. pennsylvanica* dsRNA 1571-bp long. Open squares, *P. pennsylvanica* dsRNA 1372-bp long. *P. pennsylvanica* dsRNAs used here possess 67% homology to *P. pyralis luc*. S2 cells were transfected essentially as described in the legend to Fig. 1.

RNases or nucleases. Thus, reduction in RNAi activity in the presence of unrelated dsRNA may partially result from possible competition for receptor nuclease proteins between dsRNA fragment with homology to the target gene and those without such homology.

3.2. RNAi in Chinese hamster cells

In contrast to invertebrates, only limited success with RNAi has been noted in mammals [14,15]. Study was thus made as to whether RNAi is effective in Chinese hamster CHO-K1 cells expressing *P. pyralis luc* transiently. As shown in Fig. 3A, *P. pyralis* luciferase activity was significantly reduced on exposing CHO-K1 cells to a large amount of dsRNA homologous to *P. pyralis luc*. Neither *Dfz2* dsRNA nor 714-bp long composite dsRNA, both possessing very low RNAi activity in *Drosophila* S2 cells (see Fig. 1), had any appreciable effect on *P. pyralis* luciferase activity in CHO-K1 cells. There was little or no reduction in *R. reniformis* luciferase activity under similar conditions (Fig. 3A) and this may be an indication that very high levels of dsRNA used here had little, if any, non-

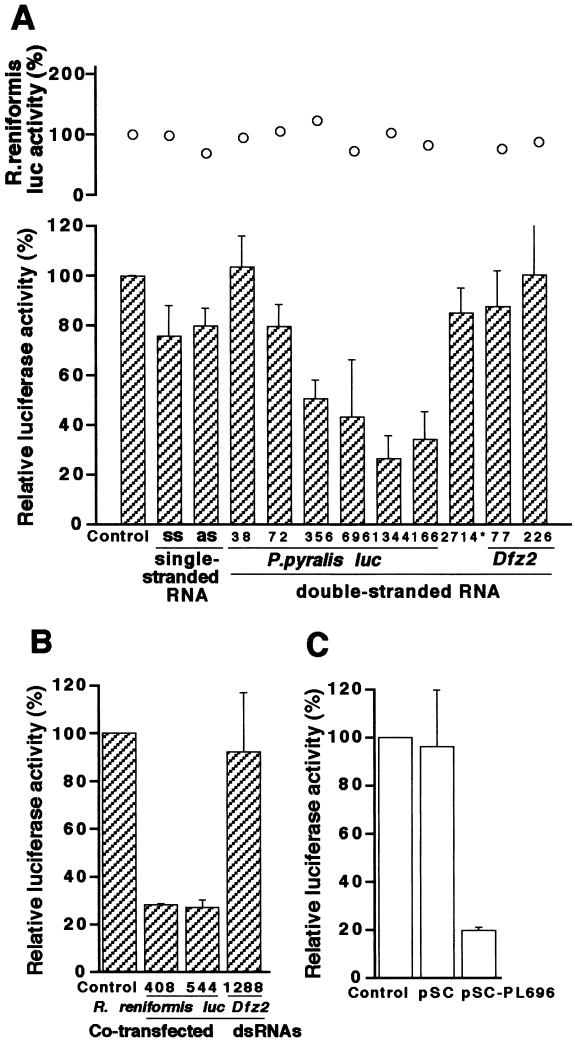

Fig. 3. RNAi in mammalian cells. A: CHO-K1 cells were transfected with 10 pmol of various sizes (38–1662 bp) of *P. pyralis luc* dsRNA, anti-sense (as) or sense (ss) single-stranded *RNA* 1344-bp long, or *Dfz2* dsRNA 226 bp in length together with 2.6 μg of pGL3-Control and 0.5 μg of pRL-TK, and the relative luciferase activity (average of three independent experiments) was presented as percentage. 10 pmol of 696 bp dsRNA showing about 50% reduction of luciferase activity corresponds to 5.0 μg, indicating that the RNAi effect in CHO-K1 cells requires 2500 times more cognate dsRNA than that in *Drosophila* S2 cells. Note that, as in the case of *Drosophila* cells, 714-bp long composite dsRNA (714*) has a very low RNAi activity, if any. Open circles, Relative *R. reniformis* luciferase activity. Note that it was not affected by the length, sequence and quantity of dsRNA exposed to cells. B: RNAi activity in CHO-K1 cells when *R. reniformis luc* was used as a target. Significant RNAi effects were observed in CHO-K1 cells co-transfected with *R. reniformis luc* dsRNA, while little or no RNAi activity was detected in CHO-K1 cells exposed by *Dfz2* dsRNA. C: RNAi induced by fold-back RNA produced by pSC-PL696. No RNAi activity could be found in CHO-K1 cells transfected with pSC.

specific effect on *R. reniformis* luciferase control. Similar RNAi effects were observed when *P. pyralis luc* was replaced with *R. reniformis luc* (Fig. 3B).

Anti-sense and/or sense single-stranded RNA contaminated in our dsRNA preparations might be responsible for reduction in luciferase activity. CHO-K1 cells were consequently exposed to cognate antisense or sense single-stranded RNA in place of cognate dsRNA but this led not to appreciable reduction of luciferase activity within the limit of error (Fig. 3A). An expression construct encoding fold-back RNA in which a pair of complementary *P. pyralis luc* sequences is in a double-helix. This construct and *P. pyralis* and *R. reniformis luc* genes were simultaneously introduced into CHO-K1 cells and transiently expressed. As with dsRNA transfection, *P. pyralis* luciferase activity was significantly reduced in a sequence-specific fashion (Fig. 3C). Thus, we conclude that cognate dsRNA introduced into or expressed in CHO-K1 cells is capable of reducing target gene activity in a sequence-specific fashion.

DsRNA required for RNAi in CHO-K1 cells was about 2500 times that for RNAi in *Drosophila* S2 cells, indicating that Chinese hamster cells possess sufficient activity to respond to exogenously added dsRNA sequence-specifically but this activity is much weaker than in *Drosophila* cells. At present, whether the molecular mechanism similar to that of *Drosophila* RNAi [17,18] is operative in CHO-K1 cell RNAi is not clear.

In mammalian cells, interferon response, triggered by dsRNA produced through viral infection [22], stimulates the formation of dsRNA-responsive protein kinase (PKR) [23] and a 2′-5′-oligoadenylated synthetase (reviewed in [24]). RNase L is activated by 2′-5′-oligonucleotides, PKR phosphorylates a translation factor to suppress overall translation and cells in an antiviral state are considered to die eventually as a result of apoptosis. However, our experiment showed that luciferase activity reduction in CHO-K1 cells persists at least until day 6 after transfection (data not shown). Thus, CHO-K1 cells exhibiting a considerable level of RNAi activity might be tolerant at least temporally to possible apoptosis due to interferon response.

Acknowledgements: We thank Dr. H. Tei for critical reading of the manuscript. This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan to K.S., and the Science Research Promotion Fund from the Promotion and Mutual Aid Corporation for Private Schools of Japan to Y.M.

References

[1] Fire, A. (1999) Trends Genet. 15, 358–363.
[2] Sharp, P.A. (1999) Genes Dev. 13, 139–141.
[3] Bosher, J.M. and Labouesse, M. (2000) Nature Cell Biol. 2, E31–E36.
[4] Napoli, C., Lemieux, C. and Jourgensen, R. (1990) Plant Cell 2, 279–289.
[5] van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N. and Stuitje, A.R. (1990) Plant Cell 2, 291–299.
[6] Ngo, H., Tschudi, C., Gull, K. and Ullu, E. (1998) Proc. Natl. Acad. Sci. USA 95, 14687–14692.
[7] Lohmann, J.U., Endl, I. and Bosch, T.C. (1999) Dev. Biol. 214, 211–214.
[8] Sanchez-Alvarado, A. and Newmark, P.A. (1999) Proc. Natl. Acad. Sci. USA 96, 5049–5054.
[9] Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998) Nature 391, 806–811.
[10] Montgomery, M.K. and Fire, A. (1998) Trends Genet. 14, 255–258.
[11] Pal-Bhadra, M., Bhadra, U. and Birchler, J.A. (1997) Cell 90, 479–490.
[12] Kennerdell, J.R. and Carthew, R.W. (1998) Cell 95, 1017–1026.
[13] Misquitta, L. and Paterson, B.M. (1999) Proc. Natl. Acad. Sci. USA 96, 1451–1456.
[14] Wargelius, A., Ellingsen, S. and Fjose, A. (1999) Biochem. Biophys. Res. Commun. 263, 156–161.
[15] Wianny, F. and Zernicka-Goetz, M. (2000) Nature Cell Biol. 2, 70–75.
[16] Tuschl, T., Zamore, P.D., Lehmann, R., Bartel, D.P. and Sharp, P.A. (1999) Genes Dev. 13, 3191–3197.
[17] Hammond, S.M., Bernstein, E., Beach, D. and Hannon, G.J. (2000) Nature 404, 293–296.
[18] Zamore, P.D., Tuschl, T., Sharp, P.A. and Bartel, D.P. (2000) Cell 101, 25–33.
[19] de Wet, J.R., Wood, K.V., DeLuca, M., Helinski, D.R. and Subramani, S. (1987) Mol. Cell. Biol. 7, 725–737.
[20] Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J. and Nusse, R. (1996) Nature 382, 225–230.
[21] Grentzmann, G., Ingram, J.A., Kelly, P.J., Gesteland, R.F. and Atkins, J.F. (1998) RNA 4, 479–486.
[22] Marcus, P.I. (1983) Interferon 5, 115–180.
[23] Clemens, M.J. (1997) Int. J. Biochem. Cell Biol. 29, 945–949.
[24] Williams, B.R.G. and Silverman, R.H. (1985) in: Clinical and Molecular Aspects of the Interferon Regulated Pathways, A.R. Liss, New York.