

# Targeted disruption of transcriptional regulatory function of p53 by a novel efficient method for introducing a decoy oligonucleotide into nuclei

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## ABSTRACT

Decoy oligonucleotides have been used for functional sequestering of transcription factors. Efficient introduction into cells is a prerequisite for the oligonucleotides to exert their blocking function. Lipofection is the most widely used technique for that purpose because of its convenience and relatively high efficiency. However, the transduction efficiency of lipofection largely depends on cell types and experimental conditions and the introduced nucleotides are not specifically directed to nuclei where they exert their major function. In the present study, we designed a new system for transporting oligonucleotides into cell nuclei. The vehicle is composed of glutathione-S-transferase, 7 arginine residues, the DNA-binding domain of GAL4 and a nuclear localization signal, which are linked with flexible glycine stretches. The p53-responsive element linked to the GAL4 upstream activating sequence was efficiently transferred by the vehicle protein into nuclei of primary cultures of neuronal cells, embryonic stem cells and various human normal cells. Transcriptional activation of p21<sup>WAF1/CIP1</sup> and Bax by p53 on exposure to cisplatin was completely blocked by introducing the p53 decoy oligonucleotide. Thus, the system developed in the present study can be a convenient and powerful tool for specifically disrupting the function of DNA-binding proteins in culture.

## INTRODUCTION

Functional inactivation of specific gene products is a powerful and promising tool not only for studying biological relevance of a given gene but also for developing target gene-based therapeutic measures. The 'loss-of-function' of a gene can be achieved by a number of different approaches, including the expression of a dominant negative form of proteins, intracellular introduction of specific antibodies, antisense oligonucleotides, siRNA and decoy oligonucleotides for DNA-binding proteins (1–4). Among those approaches, the decoy oligonucleotide method is advantageous in terms of promptness of effects, simplicity and easiness in handling (5,6).

In cells, gene expression is principally regulated at the transcriptional level by DNA-binding proteins known as transcription factors. Introduction of an excess amount of a DNA segment harboring a *cis*-element into cells results in functional sequestering of the counterpart-transacting factor from acting on the endogenous target promoters. Thus, decoy oligonucleotides, including those for NF- $\kappa$ B, E2F and AP-1, have been successfully used *in vivo* as well as *in vitro* (7–13).

Many attempts have been made to improve the efficiency of the decoy oligonucleotide method. Phosphothioation was shown to stabilize decoy oligonucleotides introduced into cells (7,14). Also, oligonucleotides became more stable when both ends were locked by adding an extra 2'-O, 4'-C-methylene bridge to the ribose ring (15). Ahn *et al.* (16) showed that circular dumbbell oligonucleotides were more resistant to exonuclease, exhibiting more prolonged effects. Another problem to be overcome is how to efficiently introduce decoy oligonucleotides into cells. The most frequently used method to introduce oligonucleotides into cells is liposome-mediated

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Labeling Kit (Amersham Biosciences) and a ULYSIS Alexa Fluor 488 Nucleic Acid Labeling Kit (Molecular Probes, Eugene, OR), respectively. Intracellular localization of the introduced molecules in living and fixed cells were observed using a fluorescent microscope (IX71-22FL/PH; CCD camera, DP50; objective lens, LCPlan F1 40 $\times$ ; Olympus) and a laser-scanning microscope (Axioplan 2; objective lens, Plan-Apocomat 63 $\times$  1.4 oil DC; Carl Zeiss MicroImaging), respectively.

### Western blot analysis

Immunoblotting was performed using a rabbit anti-GST antibody (Amersham Biosciences), a mouse anti-GAL4 (DB) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a mouse anti-human p53 antibody (Santa Cruz Biotechnology), a rabbit anti-human p21<sup>WAF1/CIP1</sup> antibody (Santa Cruz Biotechnology), a rabbit anti-human Bax antibody (Upstate Biotechnology, Lake Placid, NY) or a mouse anti-human tubulin antibody (Sigma), followed by the application of a horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (Cell Signaling Technology Inc., Beverly, MA). Positive signals were visualized using a chemiluminescence system (ECL plus, Amersham Biosciences).

### Northern blot analysis

Total RNA was isolated by the acid guanidinium thiocyanate/phenol-chloroform method. Northern blot analysis was performed under conventional conditions. Briefly, 20  $\mu$ g RNA of each sample was fractionated in a 1.0% agarose gel and transferred to a Nytran Plus nylon membrane (Amersham Biosciences). Probes synthesized from entire cDNAs of human p21<sup>WAF1/CIP1</sup> and GAPDH were used.

### Electrophoresis mobility shift assay

Electrophoresis mobility shift assay (EMSA) was performed under conditions similar to those described by Nakano *et al.* (25). Briefly, <sup>32</sup>P-labeled decoy DNA probes (0.5  $\mu$ g) were incubated with 0.1–2  $\mu$ g of crude nuclear extracts of HCT116 or PC3 cells in a reaction mixture (20  $\mu$ l) containing 60 mM KCl, 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 1  $\mu$ g poly(di-dC), 1 mM DTT, 5% glycerol and 20 mM HEPES, pH 7.9. For a supershift assay, 2  $\mu$ g of purified vehicle proteins and/or 0.5–2  $\mu$ g of a mouse anti-human p53 antibody (Santa Cruz Biotechnology) were added to the reaction mixture. DNA-protein complexes were then separated by electrophoresis in a 5% polyacrylamide gel under non-denaturing conditions.

### Chromatin immunoprecipitation assay

Chromatin immunoprecipitation was performed according to a method described previously (26). In brief, HCT116 cells were cross-linked by adding formaldehyde to 1% final concentration, and p53–DNA complex was immunoprecipitated with a mouse anti-human p53 antibody (Santa Cruz Biotechnology). A p53-responsive region in the p21<sup>WAF1/CIP1</sup> promoter was amplified using a forward primer, 5'-gtgcagacagcagtgggcttagag-3' and a reverse primer, 5'-cagaaccaggcttgaggcagctac-3'.

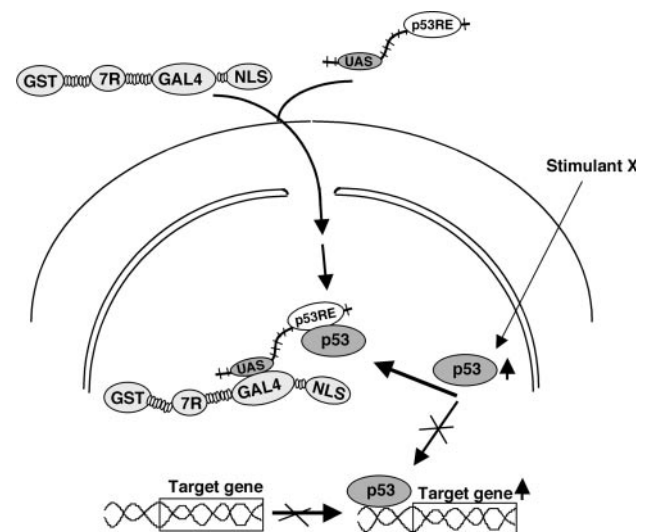
## RESULTS

### Design of experiments

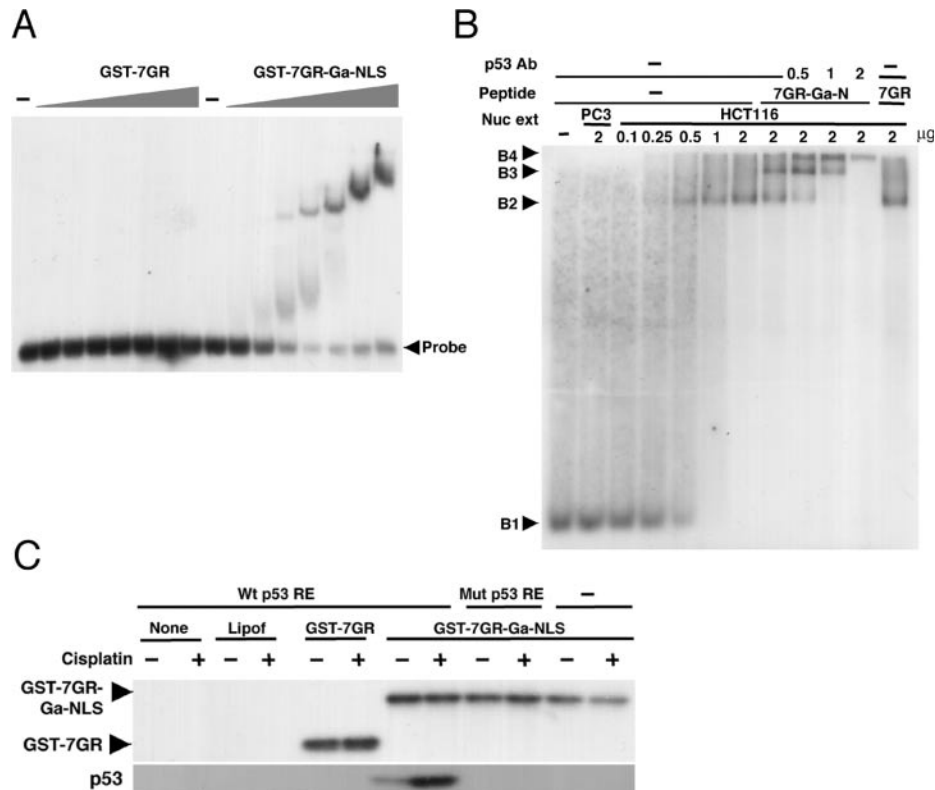
Figure 1 shows the design of a method to efficiently introduce a decoy oligonucleotide into nuclei. Two tandem p53-responsive elements (p53RE) for sequestering p53 protein were linked to the yeast UAS sequence, which binds to GAL4 in the vehicle protein. The vehicle protein (GST-7GR-Ga-NLS; 45.8 kDa) is composed of GST, 7G residues, 7 arginine residues, 7 glycine residues, the DB of GAL4, 3 glycine residues and the NLS from the N-terminal side. The poly-arginine and NLS facilitate the incorporation of proteins into cells and nuclei, respectively (23,27,28). GST is used for the purification of the recombinant proteins and also for promoting cellular incorporation of proteins (Figure 4B). The glycine stretches provide flexible linkages between each domain. The oligonucleotide was expected to be efficiently transferred to nuclei by the help of the vehicle protein, resulting in the inhibition of p53 function by sequestering it.

### Specific binding of the decoy oligonucleotide to p53

We first examined whether the decoy oligonucleotide binds to the vehicle protein and p53. When the labeled oligonucleotide was incubated with the vehicle protein GST-7GR-Ga-NLS *in vitro*, it dose dependently bound to the protein as demonstrated by EMSA, whereas it showed no binding activity to the protein lacking GAL4 and NLS domains (GST-7GR) (Figure 2A). The oligonucleotide could simultaneously bind to p53 protein in a nuclear extract prepared from HCT116 cells as well as to the vehicle protein (Figure 2B). Specificity of the binding was confirmed by supershift of the band observed when an antibody against p53 was added to the reaction



**Figure 1.** A schematic diagram of the method to introduce a decoy oligonucleotide into nuclei. The oligonucleotide is composed of UAS and two p53-responsive elements in tandem (p53RE). The vehicle protein (GST-7GR-Ga-NLS) is composed of GST, 7 glycine residues, 7 arginine residues (7R), 7 glycine residues, the DNA-binding domain (DB) of GAL4, 3 glycine residues and an NLS. UAS is for binding to GAL4 in the vehicle and p53RE sequesters p53 protein. 7R and GST promote the incorporation of the protein into cells. GST is also used for purification of the protein.



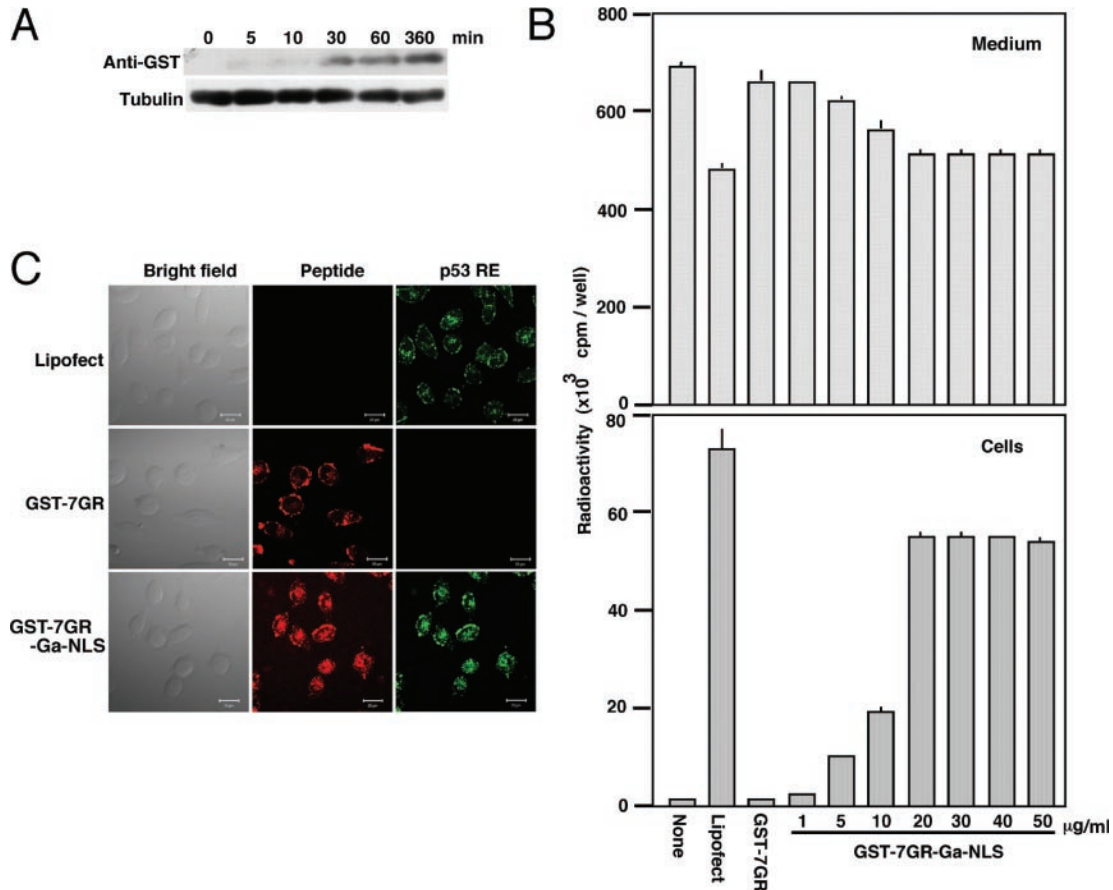
**Figure 2.** Binding of the decoy oligonucleotide to the vehicle protein. (A) Binding of the decoy oligonucleotide was assessed by EMSA. Labeled decoy nucleotide was incubated with increasing amounts of the vehicle peptide described in the legend to Figure 1 (GST-7GR-Ga-NLS) or that lacking GAL4 and NLS (GST-7GR) and analyzed by electrophoresis. (B) Binding of the decoy nucleotide not only with the vehicle protein but also with p53 protein in a nuclear extract. Labeled decoy nucleotide was incubated with indicated proteins and increasing amounts of nuclear extract prepared from p53-proficient HCT116 cells. In some tubes, an antibody against p53 was added. Arrowheads indicate the following positions: B1, the probe only; B2, the probe bound to p53; B3, the probe bound to p53 and the vehicle protein; and B4, the probe bound to p53, the vehicle protein and p53 antibody. 7GR-Ga-N and 7GR correspond to GST-7GR-Ga-NLS and GST-7GR, respectively. (C) Binding of the decoy oligonucleotide with p53 in cells. The decoy oligonucleotide (wild-type or mutated) was applied by means of lipofection (Lipof), GST-7GR or GST-7GR-Ga-NLS to HCT116 cells treated with cisplatin (10  $\mu$ g/ml, 6 h) or not treated. Introduced proteins were recovered from cell extracts using glutathione beads and analyzed by western blotting using an antibody against GST (upper panel) or an antibody against p53 (lower panel).

mixture and also by the absence of the band shift when incubated with a nuclear extract from p53-deficient PC3 cells. To examine whether the oligonucleotide bound to p53 protein within cells, the oligonucleotide was introduced into HCT116 cells by means of lipofection, GST-7GR, or GST-7GR-Ga-NLS, and the vehicle proteins recovered using glutathione beads from cell extracts were analyzed by western blotting (Figure 2C). p53 protein was detected only when the oligonucleotides containing the wild-type p53RE were applied with GST-7GR-Ga-NLS. These results indicate that the oligonucleotide designed in the present study can simultaneously bind to p53 as well as the vehicle GST-7GR-Ga-NLS *in vitro* and in cells.

#### Efficient introduction of the decoy oligonucleotide into different types of cells, particularly into nuclei

When GST-7GR-Ga-NLS was applied to HCT116 cells, the vehicle protein was first detected in the cells at 30 min and the amount slightly increased up to 360 min after the application (Figure 3A). Incorporation of the labeled oligonucleotide by GST-7GR-Ga-NLS depends on the dose of the vehicle protein as assayed at 7 h after application (Figure 3B). Twenty micrograms of GST-7GR-Ga-NLS was sufficient to attain the

maximum introduction to  $10^4$  HCT116 cells cultured in 1 ml of medium. Under these conditions,  $\sim 10\%$  of the 200 ng oligonucleotide added to the medium was incorporated into the cells. Although lipofection could also efficiently introduce the oligonucleotide into the cells, most of the incorporated oligonucleotides were found in the perinuclear cytoplasm (Figure 3C, top panels) in accordance with the report by Bene *et al.* (17). In contrast, the oligonucleotide was detected mostly in nuclei when introduced using GST-7GR-Ga-NLS (Figure 3C, bottom panels). The preferential introduction of the decoy oligonucleotide into nuclei by GST-7GR-Ga-NLS in comparison with lipofection was also observed in NHK cells and mouse ES cells (Figure 4A). Applicability of the present method to a wide variety of cells was confirmed by western blotting (Figure 4B) and by the determination of radioactivity after the application of the labeled oligonucleotide (Figure 4C). GST-7GR-Ga-NLS facilitated the incorporation of the oligonucleotide in primary cultures of mouse and rat brain neuronal cells and rat hepatocytes, in normal human keratinocytes, fibroblasts (OUMS-36) and leukocytes, and in mouse ES cells. Efficiency of the cellular incorporation was not affected by cell density in OUMS-36 (Figure 4C, lower right panel). Either GST or 7-arginine stretch alone could not promote the protein transduction (Figure 4B).



**Figure 3.** Introduction of the decoy nucleotide into cells by the vehicle protein. (A) Time course of cellular uptake of the vehicle protein. GST-7GR-Ga-NLS (20 µg) was applied to HCT116 cells and was recovered from cell extracts using glutathione beads at various times after the application. (B) Introduction of the decoy oligonucleotide into HCT116 cells by the vehicle proteins. Radioactivity in cells and medium was determined 6 h after the application of labeled decoy oligonucleotide to HCT116 cells by lipofection (Lipofect), GST-7GR (50 µg/ml) or indicated amounts of GST-7GR-Ga-NLS. (C) Introduction of the decoy oligonucleotide into cell nuclei. The decoy oligonucleotide labeled with Alexa Fluor 488 (in green) was applied to HCT116 cells with the vehicle proteins labeled with Cy3 (in red) and observed 6 h after application. The cells were observed by a confocal laser microscope at a plane horizontally cutting in the middle part of cells.

GST-7GR-Ga, a vehicle protein lacking NLS, hardly transported oligonucleotides into nuclei (data not shown).

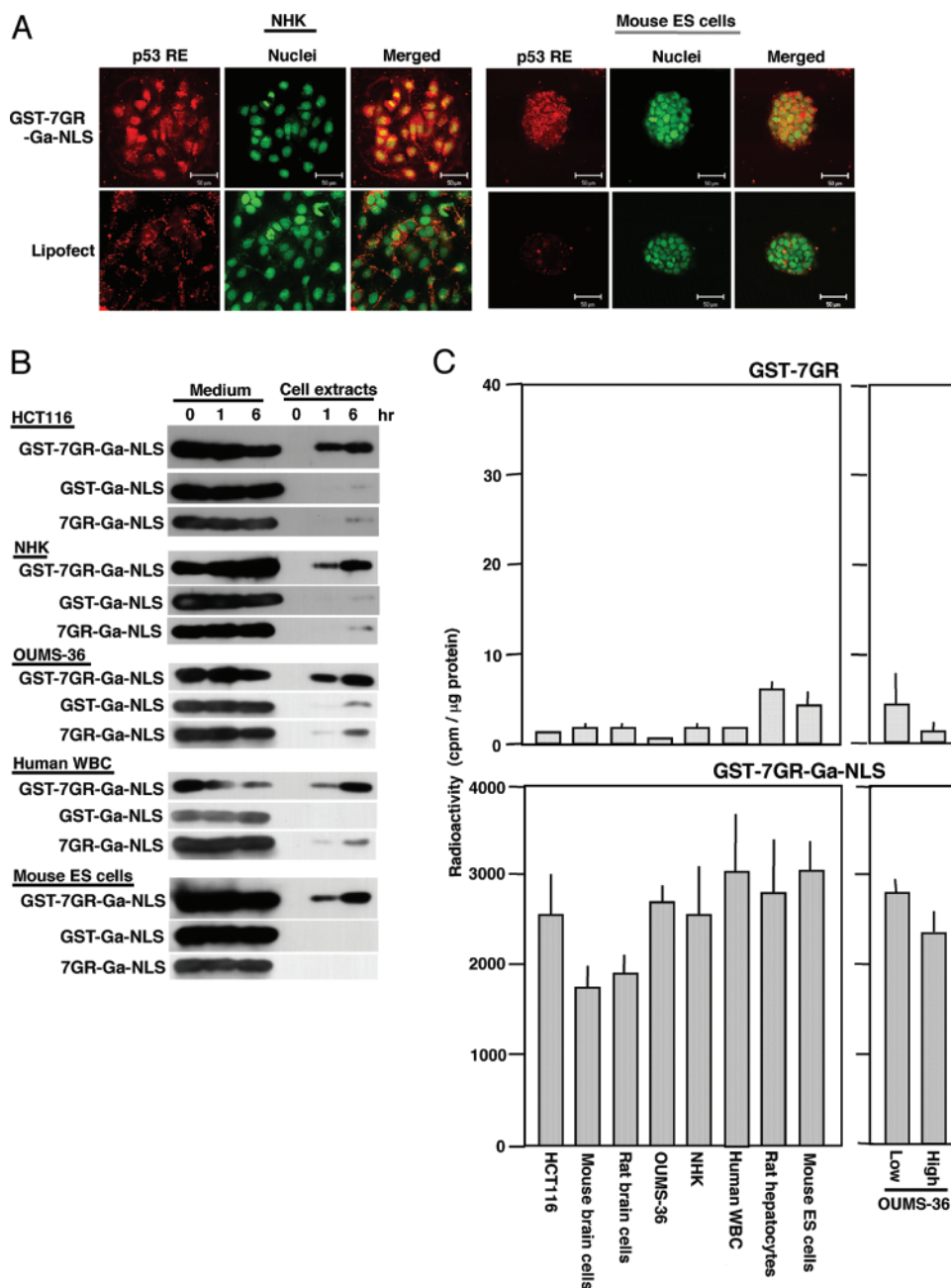
### Inhibition of the function of p53

Biological relevance of the decoy oligonucleotide introduction was studied by examining the activity of p53 in transcriptional regulation. The wild-type or mutated decoy oligonucleotide was applied to HCT116 cells with GST-7GR-Ga-NLS. One hour later, cisplatin (10 µg/ml) was added to the medium and the cells were harvested for analysis 6 h after the oligonucleotide application. By treatment with cisplatin, the protein level of p53 was elevated and this was not affected by any treatments for the introduction of oligonucleotides (Figure 5A). In accordance with the reported findings that the p21<sup>WAF1/CIP1</sup> and Bax promoters were activated on exposure to cisplatin (29,30), we detected increased amounts of p21<sup>WAF1/CIP1</sup> and Bax proteins in the cells treated with cisplatin. The induction of both proteins was completely suppressed when the cells were pretreated with the wild-type oligonucleotide and GST-7GR-Ga-NLS (Figure 5A). To obtain some mechanistic insights, we examined binding of p53 to the p21<sup>WAF1/CIP1</sup> promoter by a chromatin

immunoprecipitation assay. p53 protein was activated to bind to the p21<sup>WAF1/CIP1</sup> promoter by treatment with cisplatin and the binding was completely suppressed when the oligonucleotide harboring the wild-type p53-responsive element, but not that having the mutated element, was introduced by GST-7GR-Ga-NLS (Figure 5B). Although lipofection could introduce the oligonucleotide with efficiency comparable with that by GST-7GR-Ga-NLS (Figure 3B), the induction of p21<sup>WAF1/CIP1</sup> and Bax protein (Figure 5A) and the binding p53 to the p21<sup>WAF1/CIP1</sup> promoter (Figure 5B) were not appreciably affected. This indicates that transportation of the decoy oligonucleotide to the nuclei and not to the cytoplasm is crucial for modulating function of transacting factors in cells.

### Stability of the decoy oligonucleotide in cells

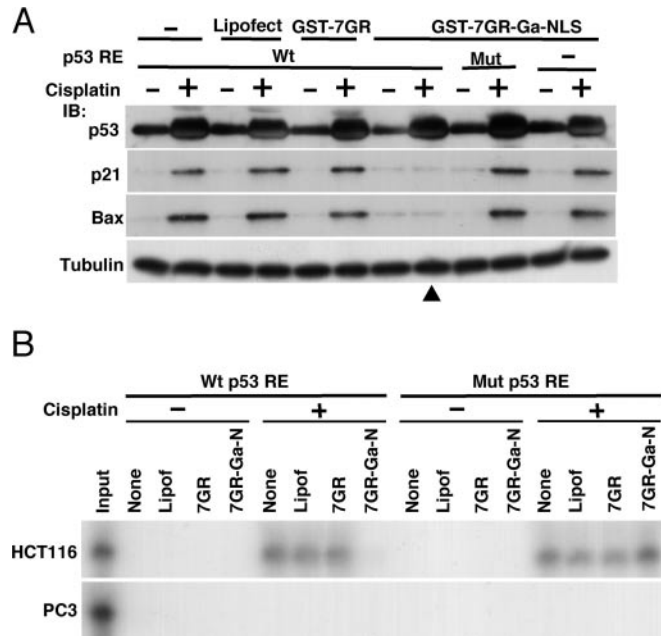
Finally, we examined stability of the incorporated oligonucleotide and duration of the functional sequestering of p53 protein. Labeled decoy oligonucleotide was introduced into HCT116 cells or NHK cells, and the radioactivity recovered from the cells was monitored by autoradiography after electrophoresis. As shown in Figure 6A, the intracellular oligonucleotide levels remained essentially unchanged until 12 h,



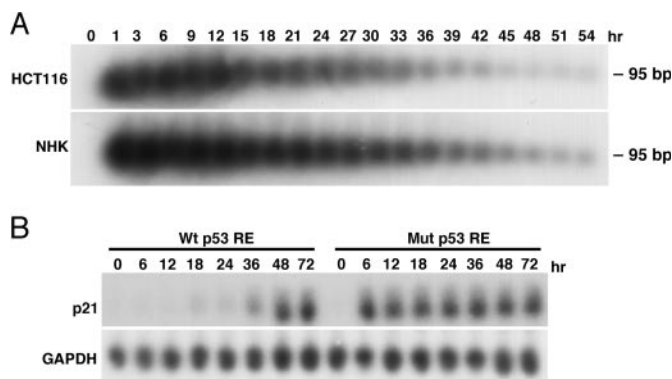
**Figure 4.** Efficient introduction of the decoy oligonucleotide into diverse types of cells. (A) The decoy oligonucleotide labeled with Alexa 546 (in red) was applied with the vehicle protein or by lipofection onto normal human keratinocytes (NHKs) or mouse ES cells and observed by a confocal laser microscope. Nuclei were stained with SYBR Green (in green). (B) Proteins of different structural compositions were applied to various types of cells, and the media and cell extracts were analyzed by western blotting using anti-GAL4 antibody. GST-7GR-Ga-NLS, the complete vehicle protein; GST-Ga-NLS, a vehicle protein lacking 7 arginine residues; 7GR-Ga-NLS, a vehicle protein lacking GST. (C) Radiolabeled decoy oligonucleotide was applied to various types of cells with the complete vehicle protein (GST-7GR-Ga-NLS) or a protein lacking GAL4 and NLS (GST-7GR), and radioactivity in cell extracts was determined. Low and high indicate low-density and high-density cultures of OUMS-36, human normal diploid fibroblasts.

gradually declined afterwards reaching a level of about half around 24 h, and decreased to an undetectable level around 48 h after the application in both cell types. Duration of the biological effects of the introduced oligonucleotide was studied in NHK cells because HCT116 cells underwent apoptotic cell death by treatment with cisplatin. NHK cells were treated with cisplatin 1 h after the introduction of the wild-type or mutated decoy oligonucleotide into the cells, and then p21<sup>WAF1/CIP1</sup> mRNA level was monitored (Figure 6B). In the

cells exposed to the mutated p53 decoy oligonucleotide, p21<sup>WAF1/CIP1</sup> mRNA was induced and remained at similar levels during the observation period of 72 h. Introduction of the wild-type p53 decoy oligonucleotide completely suppressed the induction of p21<sup>WAF1/CIP1</sup> mRNA up to 24 h. A low level of p21<sup>WAF1/CIP1</sup> mRNA was detected at 36 h and the amount had increased to a level comparable with that of untreated cells at 48 h. This kinetics accorded well with the amount the decoy oligonucleotide in the cells (Figure 6A).



**Figure 5.** Biological effects of introduction of the decoy oligonucleotide. (A) A decoy oligonucleotide carrying wild-type p53RE or mutated p53RE was introduced into HCT116 cells with or without cisplatin treatment. Cell extracts prepared 6 h after the application were analyzed for indicated proteins by western blotting. Arrowhead indicates the critical lane showing suppression of the function of p53. (B) Chromatin immunoprecipitation assay for p53 protein bound to the p21<sup>WAF1/CIP1</sup> promoter. The cells were treated under conditions similar to those described in (A) and immunoprecipitates with anti-p53 antibody were subjected to PCR amplification using primers covering p53 RE in the p21<sup>WAF1/CIP1</sup> promoter. PC3 was used as a negative control because of its deficiency of p53. 7GR-Ga-N and 7GR correspond to GST-7GR-Ga-NLS and GST-7GR, respectively.



**Figure 6.** Stability of the decoy oligonucleotide in cells. (A) Radiolabeled decoy oligonucleotide was applied with GST-7GR-Ga-NLS to HCT116 or NHK cells and amounts of the intact oligonucleotide (95 bp in length) recovered from the cells was monitored by electrophoresis and autoradiography. (B) Northern blot analysis for p21<sup>WAF1/CIP1</sup> mRNA after the introduction of the oligonucleotides. NHK cells were subjected to application of the wild-type or mutated decoy oligonucleotide and GST-7GR-Ga-NLS at the time point 0, exposed to cisplatin (10  $\mu$ g/ml) from 1 h later and harvested at indicated time points. GAPDH was used as a control for applied amounts of RNA.

## DISCUSSION

In the present study, we designed a vehicle protein for efficient introduction of decoy nucleotides into cell nuclei. It is com-

posed of GST, 7 arginine residues, the DB of GAL4 and the NLS of SV40 large T antigen, which are linked with flexible glycine stretches. The present system has the following advantages:

- (i) It is applicable for a broad variety of cells, including primary cultured cells and ES cells (Figure 4B and C).
- (ii) It is highly efficient in terms of transduced cell rate (practically 100%) (Figures 3C and 4A).
- (iii) It is efficient in terms of targeting the oligonucleotide for cell nuclei (Figure 3C), which is a prerequisite for interfering with transcriptional activation (Figure 5A and B).
- (iv) It is prompt and efficient in exhibiting the interfering function (Figures 3A and 5A).
- (v) It is applicable for other DNA-binding proteins whose target elements are known.
- (vi) It is easy to use and economical. The recombinant vehicle protein can be produced in a large amount and easily purified and stocked.

In most studies on the effect of decoy oligonucleotides, lipofection has been used for transducing oligonucleotides into cells. Efficiency of transfection by lipofection is high compared with the efficiencies of other DNA transfection methods. However, the efficiency largely depends on cell types. Another potential shortcoming of lipofection is that it does not directly help nuclear translocation of the oligonucleotide, which is critical for interfering with the function of transcription factors (17). In the present study, we attempted to actively transport oligonucleotide into nuclei. As shown in Figure 3C, nearly half of the incorporated oligonucleotide was found in the nuclei, a much larger proportion than that observed in cells transduced by lipofection.

The GST and 7 arginine residues cooperatively facilitate to introduce the protein into cells (Figure 4B). Namiki *et al.* (31) reported that the fluorescent-dye-labeled GST from *Schistosoma japonicum* penetrated into mammalian cells even when conjugated to a short-peptide load. In the present system, GST derived from yeast alone could not promote the protein transduction (Figure 4B). A number of different molecules are known to transduce proteins into cells. TAT domain derived from human immunodeficiency virus (32) and 11-arginine stretch (27,28) are widely used for protein transduction. Covalent conjugation of proteins with polyethylenimine is another potent measure for protein transduction (33,34). The strong cation charge of polyethylenimine, however, results in non-specific binding to DNA and is inappropriate for the present purpose.

p53 is known to exert its transactivation capacity through forming a homotetramer (35). At present, it is not clear whether p53 protein formed a tetramer complex in the presence of the decoy oligonucleotides under the present conditions. Since the DNA-binding domain resides in the central part of p53 protein and is apart from the C-terminal tetramerization domain, the p53 protein bound with the oligonucleotides could still form homotetramers. However, the vehicle protein that most likely remains combined with the oligonucleotides may interfere with the association of p53 protein. Chan *et al.* (36) showed that a mutant p53 defective in DNA binding, unlike p53 mutated in the transactivation domain, was inefficient in exhibiting the dominant negative effect. This indicates inefficiency in inhibiting the

transactivation capacity of p53 by blocking its DNA-binding activity. The number of p53 molecules per cell was estimated to be several hundred thousand at most (37). In the present study, ~10% of the applied amount of the oligonucleotide was incorporated into cells (Figure 3). At the lowest estimate, about one hundred excess molecules of the oligonucleotide were present in cell nuclei in comparison with that of p53 protein, being sufficient to completely sequester the transacting factor.

The present method can be used not only for studying the functions of transacting factors but also the function of any proteins that exert their functions through binding to a DNA region in a sequence-specific manner. It can also be used for the determination of DNA elements relevant to transcriptional regulation among candidate segments in the promoter region. With the features described, the present method could certainly be a convenient and powerful tool for studying the functions of DNA-binding proteins in culture, though not suitable for using *in vivo*.

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*Conflict of interest statement.* None declared.

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