BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **240,** 203–207 (1997)
ARTICLE NO. RC977563

Inhibition of Gene Expression from the Human c-*erb*B Gene Promoter by a Retroviral Vector Expressing Anti-gene RNA

Takashi Okada,*[,1] Hiroshi Amanuma,† Yoshie Okada,* Masahiro Obata,† Yutaka Hayashi,* Kazuo Yamaguchi,‡ and Junkoh Yamashita*

**Department of Neurosurgery, Kanazawa University School of Medicine and ‡Institute for Gene Research, Kanazawa University, Kanazawa, Japan; and †Laboratory of Gene Technology and Safety, Tsukuba Life Science Center, The Institute of Physical and Chemical Research (RIKEN), Tsukuba, Ibaraki, Japan*

Received September 23, 1997

Anti-gene is a potent inhibitor of transcriptional promoter activity and subsequent gene expression. This property has been exploited to suppress the expression of a variety of oncogenes for regulating tumor proliferation or viral activities. In this paper, we describe a novel retroviral vector designed to express human c-*erb*B anti-gene RNA and to reduce the promoter activity in the cells. Mouse fibroblast NIH3T3 cells were stably transfected with an expression construct containing a truncated human c-*erb*B gene promoter fused to the firefly luciferase reporter gene. Infection into these cells of the c-*erb*B anti-gene retroviral vector targeted to the 26 bp pyrimidine-rich element in the human c-*erb*B gene promoter resulted in a dose-dependent decrease in the luciferase activity of the cells. Retroviral vector expressing anti-gene RNA may be useful as an alternative program of gene regulation in the cells.

Nucleic acid triplexes were first described over 30 years ago (1, 2), and the structures of some of them have been determined at high resolution (3, 4). Since the number of copies of DNA is much fewer than those of transcribed mRNA, anti-gene oligonucleotides targeted against the promoter region would have an advantage over antisense oligonucleotides with an mRNA as a target. DNA triplexes inhibited transcription of the c-*myc* gene (5) and blocked binding of the Sp1 transcription factor to its target sequence in SV40 DNA in cell-free systems (6). We previously reported that oligonucleotides designed to form a triple helix with enhancer elements in the human c-*erb*B gene promoter suppressed the c-*erb*B mRNA formation and also the proliferation of human glioma cell lines (7). Mixed purine-pyrimidine oligodeoxynucleotides were designed to form collinear DNA triplexes with pyrimidine-rich elements in the c-*erb*B gene promoter. These results presented availability of anti-genes as negative mediators of transcription and subsequent gene expression. In glioblastoma multiforme, the most commonly affected oncogene is the c-*erb*B gene (8, 9), which is located on chromosome 7 and codes for a transmembrane receptor protein, the epidermal growth factor receptor (EGFR) with protein tyrosine kinase activity. We have been exploring a novel form of anti-gene strategy intended to suppress the expression of the EGFR for *in vivo* application.

RNA has been suggested to be useful for targeting double helical DNA. Since the free energies of binding of D (DNA) and R (RNA) to double helical DNA (DD) are comparable, R and D were shown to bind DD with similar affinities (10), while Roberts and Crothers found R formed a more stable complex with DD than D (11). These results suggested that in vivo targeting of double helical DNA with RNA may be possible. However, efficiency of the anti-gene RNA as an inhibitor of transcription has not yet been proven.

Genetically altered viruses are able to deliver genes efficiently into tumor cells. Retrovirus-mediated gene transfer has been applied to the treatment of malignant glioma in animal models (12-19). Since retroviruses infect actively dividing cells but not non-dividing cells, it is possible to transfer the gene selectively into glioma cells using retroviral vectors without exerting toxicity on normal neurons and astrocytes (20). We report here that a retroviral vector expressing an anti-gene RNA designed to interact with pyrimidine-rich elements of the human c-*erb*B gene promoter could sup-

[1] To whom correspondence should be addressed at present address: Clinical Gene Therapy Branch, National Human Genome Research Institute, NIH Bldg.10 - Room 10C103, Bethesda, MD 20892-1852. Fax: (301) 496-7184. E-mail: tokada@nhgri.nih.gov.

0006-291X/97 $25.00

press expression of the reporter gene which is driven by the c-*erb*B gene promoter.

MATERIALS AND METHODS

Plasmid construction. All plasmids were constructed by the standard recombinant DNA techniques (21). Based on the previous results using the synthetic oligodeoxyribonucleotides (7), the pLNCA containing the internal CMV promoter fused to the human c-*erb*B anti-gene was constructed (Fig. 1B). The double-stranded oligodeoxyribonucleotide representing the anti-gene sequence (EGFR26-2) to the pyrimidine-rich 26 bp sequence (−109 to −84 region) in the human c-*erb*B gene promoter containing the nuclear factor Sp1 binding site (−99 to −90) and the transcription start site (Fig. 1A) was synthesized with additional *Hin*d III and *Cla* I sites at each end, and inserted between the *Hin*d III and *Cla* I sites of pLNCX (22). A diagram is shown in Fig. 1C for the structures of pLNCL and pLNEL. The firefly luciferase cDNA fragment from the plasmid JD206 (23) was subcloned at the *Hin*d III and *Cla* I sites of pLNCX to produce pLNCL. The human c-*erb*B gene promoter fragment (−459 to −1 region) obtained by PCR using pEP5 (24) as a template replaced the CMV promoter of pLNCL by making use of the *Bam*H I and *Hin*d III sites to result in pLNEL. Previous deletion analysis of the human c-*erb*B gene promoter indicated that the region from −167 to −105 was important for the maximal promoter function (25).

Cell culture, transfection and production of retroviral vector. Mouse embryonic fibroblast NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS). The cells were maintained at 37°C in a humidified atmosphere of 5 % CO_2/95 % air. Ecotropic packaging cell line psi-CRE (26) was also grown in DMEM containing 10 % FBS. Transfection of pLNCL and pLNEL into NIH 3T3 cells by the calcium phosphate co-precipitation method was performed as described previously (27), and stable transformants were selected with G418 (Gibco) at a concentration of 0.5 mg/ml. Independent colonies were isolated and expanded. Likewise, transfection of pLNCX and pLNCA into psi-CRE cells was performed, and stable transformants were selected with G418. Independent colonies were isolated and expanded, and their culture supernatants served as sources of infectious retroviral vectors LNCX and LNCA. The vector virus-containing medium was centrifuged at 30,000 × *g* for 20 min to remove cells and debris, then the retroviral vector was concentrated by further centrifugation at 30,000 × *g* for 6 hr to a titer of 1×10^7 $G418^r$ colony forming units (cfu)/ml (28) and used to infect cells.

c-erbB anti-gene RNA expression in target cells. Forty-eight hrs after adding the anti-gene retroviral vector LNCA, total RNA was extracted from the 3T3/LNEL and 3T3/LNCL cells by acid guanidium thiocyanate-phenol-chloroform extraction method (29). Expression of the c-*erb*B anti-gene RNA was detected by the reverse transcription-polymerase chain reaction (RT-PCR) (GeneAmp Thermostable rTth reverse transcriptase RNA PCR kit, Perkin Elmer Cetus). Sense and antisense primers for c-*erb*B anti-gene were 5′-AAGCTTGGCGGCGAGGCGGGGACTCGGGCGGAATCGAT-3′ and 5′-CTGCTTACCACAGATATCCTGTTTGGCCC-3′, respectively. The former corresponded to the anti-gene sequence, and the latter to the U3 portion of the 3′ long terminal repeat (LTR). The reverse transcriptase reaction was performed at 70°C for 10 min and conditions for PCR consisted of initial denaturation at 94°C for 2 min, and 40 cycles of denaturation at 95°C for 1 min and annealing and extention steps at 60°C for 1 min. The PCR products were analyzed by electrophoresis on a 2 % agarose gel with ethidium bromide staining. Furthermore, the PCR product was cloned into the pGEM-T vector (Promega) and the nucleotide sequence was determined by ALF DNA sequencer (Pharmacia).

S1 nuclease protection assay. LNCA vector-derived c-*erb*B anti-gene RNA was characterized by the S1 nuclease protection assay. A 340-bp *Sty* I / *Cla* I restriction fragment derived from pLNCA (Fig. 1B) was inserted into the pBluescript SK(+) and linealized by *Not* I digestion. A 410-base single-stranded DNA probe was synthesized and labeled with [α-^{32}P]dCTP using the Prime-a Probe DNA labeling kit (Ambion, Texas). An excess of probe was hybridized with 10μg of total RNA extracted from the 3T3/LNEL cells which had been infected with the LNCA vector or with 10μg of yeast RNA at 42°C for 12hr. The hybridization products were digested with S1 nuclease at 250 units/ml at 37°C for 30 min (S1-Assay, S1 nuclease protection assay kit, Ambion, Texas). The digestion products were seperated in a 15 cm long 5% polyacrylamide gel containing 8 M urea and detected by autoradiography.

Luciferase assay. The 3T3/LNEL and 3T3/LNCL cells (1×10^4 cells) were plated in 48-well tissue culture plates 24 hr before addition of the retroviral vector LNCA at multiplicity of infection (moi) of 5 or 10. They were exposed to the culture supernatant containing the retroviral vector and 4 μg/ml polybrene at 37°C for 4 hr. Then the medium was replaced with a fresh virus-free medium, and the cells were cultured for 48 hr at 37°C. Cell extracts were prepared and assayed for the luciferase activity using the enhanced luciferase assay kit (Pica Gene, Wako, Tokyo). Briefly, the monolayer cells were washed three times with phosphate buffered saline (PBS) and extracted with 200 μl of 1 × cell lysis buffer for 15 min at room temperature. Fifty μl of the lysates were analyzed using a luminometer K-100 (Kikkoman, Tokyo) with 50 μl injections of substrate. Luciferase activity was reported as relative light units (RLU). All assays were carried out in triplicate. The cell lysates were also assayed for protein content as described previously (30).

RESULTS AND DISCUSSION

In order to examine the effect of anti-gene RNA expression retroviral vector LNCA on the human c-*erb*B gene promoter activity in the cell, an expression construct containing the human c-*erb*B gene promoter (from −459 to −1) fused to the luciferase reporter gene (pLNEL) and that containing the CMV promoter as a control (pLNCL) were stably transfected into the mouse fibroblast cell line NIH 3T3. After drug selection of the transfected cells, clones LNEL-11 (3T3/LNEL) and LNCL-10 (3T3/LNCL) were found to express the highest luciferase activity and used for all the subsequent experiments.

Expression of the human c-*erb*B anti-gene RNA in target cells (3T3/LNEL and 3T3/LNCL) was examined by the RT-PCR method 48 hr after infection of the retroviral vector LNCA at moi of 5. Total RNA was extracted from cells and used as a template for RT-PCR. After 40 cycles of amplification, PCR products were analyzed on a 2 % agarose gel (Fig. 2). In both the 3T3/LNEL and 3T3/LNCL cells, a RT-PCR product of the expected size (242 bp) was detected while uninfected controls did not show the product (data not shown), indicating expression of the anti-gene RNA in these cells.

In order to characterize the LNCA vector-derived transcripts containing the anti-gene sequence in the 3T3/LNEL cells infected with the LNCA vector, S1 nuclease protection analysis was carried out. Structure of the LNCA vector genome (Fig. 1B) predicted the presence of 5′ LTR- and internal CMV promoter-regu-

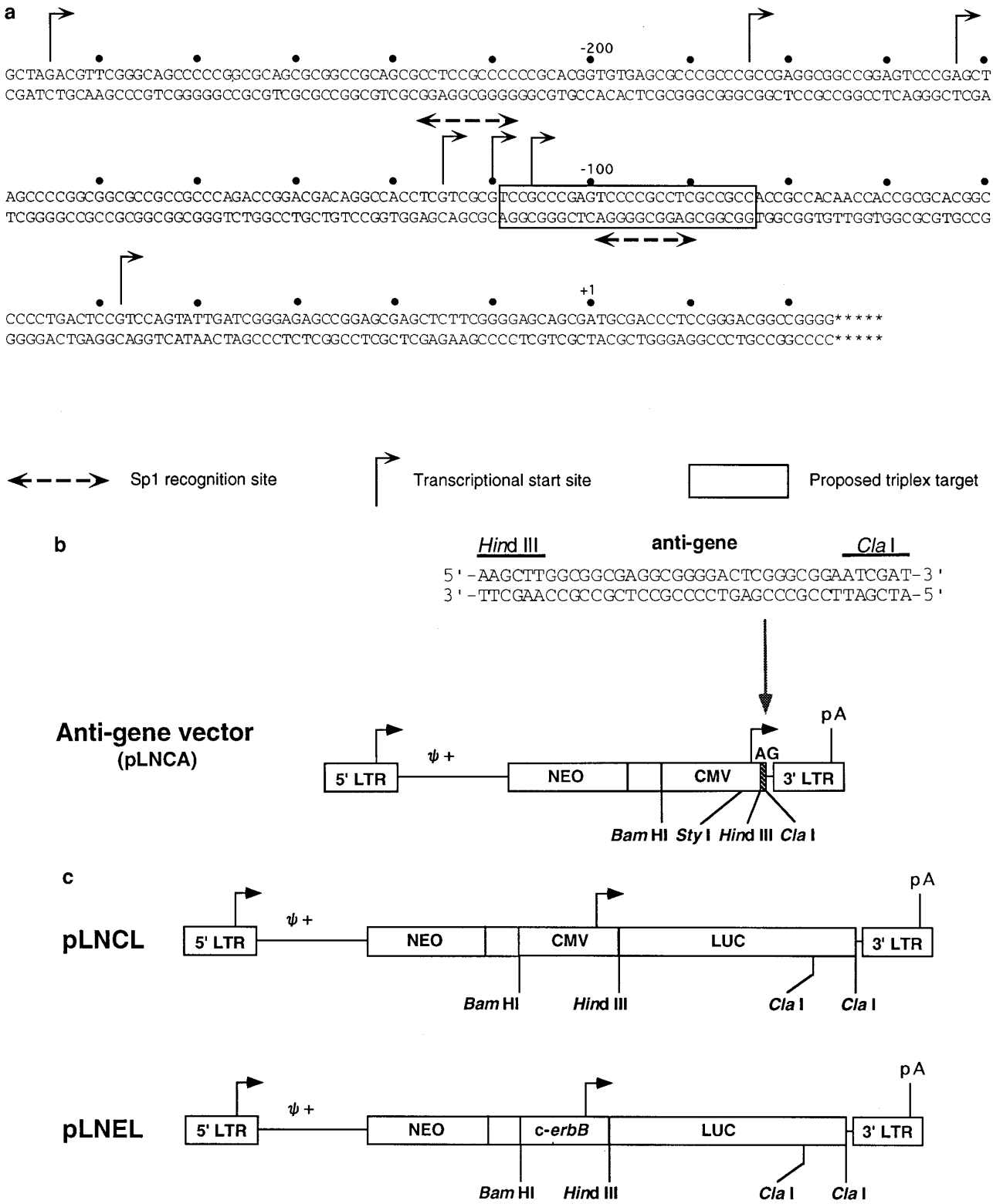

FIG. 1. Structures of the recombinant plasmids used in this study. (A) The 26-mer anti-gene sequence is antisense to the portion (−109 to −84, boxed) of the human c-*erb*B gene promoter. Nucleotide sequence, Sp1 recognition sites, and transcriptional start sites are from the previously published report (Johnson et al., 1988). Nucleotide +1 indicates the translational start site. (B) Synthetic double-stranded oligodeoxyribonucleotide representing the anti-gene sequence (AG) and the *Hin*d III and *Cla* I restriction enzyme sites at either end was inserted between the *Hin*d III and *Cla* I sites of pLNCX to result in the anti-gene vector pLNCA. (C) Luciferase-expression plasmid vectors pLNCL and pLNEL. The neomycin resistant gene (NEO) is driven by the 5′ LTR, whereas the luciferase gene (LUC) is driven by the cytomegalovirus immediate early promoter (CMV) or the human c-*erb*B gene promoter. The human c-*erb*B gene promoter fragment (−459 to −1 region) replaced the CMV promoter of pLNCL by making use of the *Bam*H I and *Hin*d III sites to result in pLNEL.

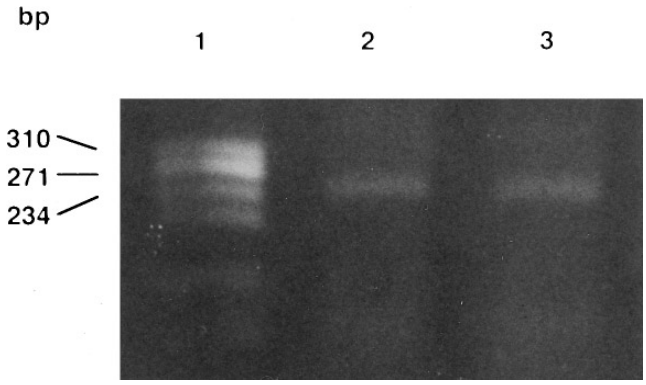

FIG. 2. Expression of the human c-*erb*B anti-gene RNA in 3T3/LNEL and 3T3/LNCL cells 48 hr after infection of the anti-gene retroviral vector LNCA. RT-PCR products were separated in a 2 % agarose gel and stained by ethidium bromide. Lane 1, *Phi* X174/*Hae* III DNA size marker; lane 2, 3T3/LNEL cells; lane 3, 3T3/LNCL cells.

lated transcripts. 5′ LTR-regulated transcripts would protect the probe from S1 nuclease digestion, giving a band of 340 bases. CMV promoter-regulated transcripts would give a band of 120 bases. Both bands were seen in the autoradiogram shown in Fig. 3. (lane B), indicating that both 5′ LTR- and internal CMV promoter-regulated transcripts were present in the 3T3/LNEL cells infected with the LNCA vector.

Luciferase activity exhibited by the 3T3/LNEL cells was sensitive to infection of the LNCA vector in a dose-dependent manner (Fig. 4A). Treatment of the 3T3/LNEL cells with the LNCA vector at moi of 5 and 10 for 48 hr resulted in a decrease of the luciferase activity to 74.2 ($P<0.001$) and 52.0% ($P<0.001$), respectively, of that exhibited by the control untreated 3T3/LNEL cells. However, treatment with the control retroviral vector LNCX under similar conditions resulted in no reduction of the luciferase activity, indicating that infection of a retroviral vector itself would be unlikely to cause reduction of the luciferase activity. In order to examine the possibility that the LNCA vector causes decreased proliferation of the NIH 3T3 cells leading to the reduced luciferase activity, the 3T3/LNCL cells

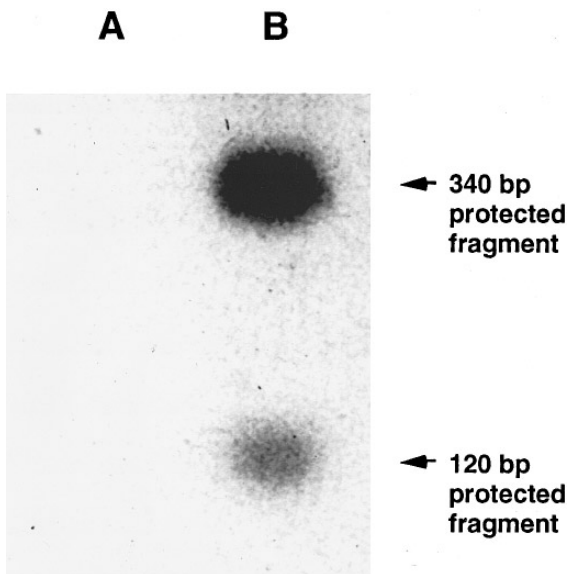

FIG. 3. Characterization of the LNCA vector-derived transcripts containing the anti-gene sequence by S1 nuclease protection analysis. Total RNA extracted from the 3T3/LNEL cells 48hr after infection of the LNCA vector was used to protect the ^{32}P-labeled 410-base single-stranded DNA probe from the S1 nuclease digestion (lane B). Lane A shows the result obtained by using yeast RNA as a negative control.

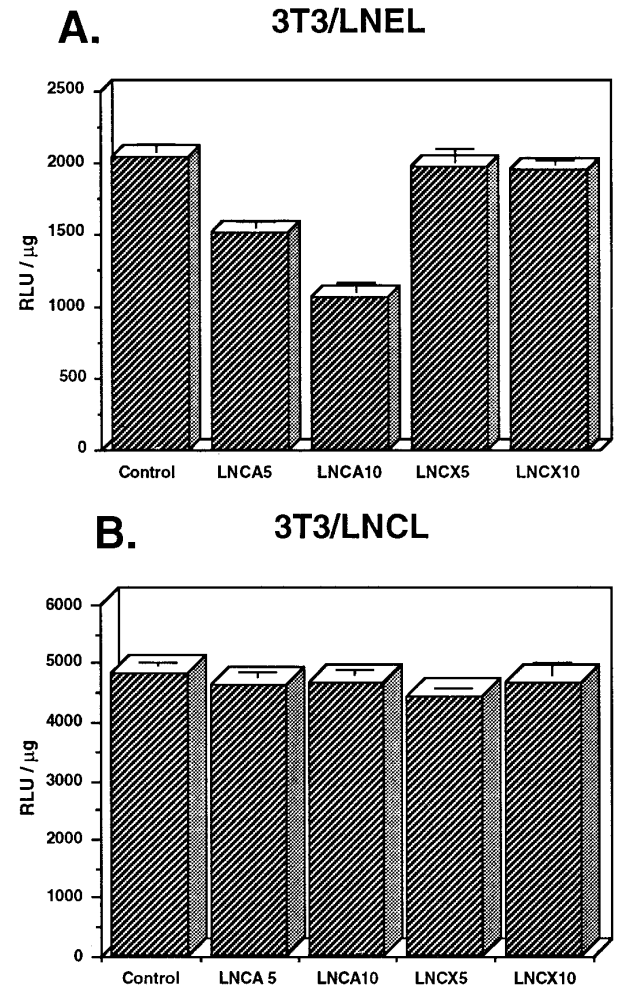

FIG. 4. Effect of infection of the human c-*erb*B anti-gene retroviral vector LNCA on the luciferase activity of 3T3/LNEL and 3T3/LNCL cells. Forty-eight hr after infection of 3T3/LNEL (A) or 3T3/LNCL (B) cells by the LNCA vector at moi of 5 (LNCA5) or 10 (LNCA10), a cell extract was prepared and its luciferase activity and protein content were measured. The specific luciferase activity is presented in the figure in comparison to that exhibited by the uninfected cells (control). Each value represents the mean ±SD (n=3). As a control, each cell was infected by the LNCX vector at moi of 5 (LNCX5) or 10 (LNCX10), and both the luciferase activity and the protein content were measured similarly.

were infected with the LNCA vector and the luciferase activity was measured. There was no reduction in the enzyme activity (Fig. 4B) in the cells infected at moi of 5 and 10.

These results suggest that the reduction of the luciferase activity in 3T3/LNEL cells treated with the LNCA vector is due to the specific effect of the transcribed human c-*erb*B anti-gene RNAs on expression of the luciferase gene. Since these RNAs harbor the 26-mer anti-gene sequence, which as a synthetic oligodeoxyribonucleotide was shown to form a triple helix with double-stranded DNA of the promoter region of the human c-*erb*B gene *in vitro* (7), it is likely that these RNAs form a triple helix with double-stranded DNA of the target promoter region and thus inhibit the initiation of transcription of the reporter luciferase gene. However, the possibility is not excluded that these RNAs may form a duplex with mRNAs of the luciferase gene transcribed from the c-*erb*B gene promoter since these RNAs are antisense to the mRNAs and some of the transcription start sites are located upstream to the 26-mer sequence (Fig. 1A) (25). Further *in vitro* as well as *in vivo* studies are necessary in order to know the detailed mechanism of suppression of the expression of the reporter gene.

ACKNOWLEDGMENTS

This work was supported in part by grants-in aid for scientific research from the Ministry of Education, Science, and Culture of Japan (07457310, 07557359). We are grateful to Masafumi Onodera MD, PhD for assistance in the S1 mapping study.

REFERENCES

1. Felsenfeld, G., Davies, D. R., and Rich, R. (1957) *J. Am. Chem. Soc.* **79,** 2023–2024.
2. Felsenfeld, G., and Rich, A. (1957) *Biochem. Biophys. Acta* **26,** 457.
3. Arnott, S., and Selsing, E. (1974) *J. Mol. Biol.* **88,** 509–521.
4. Helene, C. (1991) *Anti-Cancer Drug Design* **6,** 569–584.
5. Cooney, M., Czernuszewicz, G., Postel, E. H., Flint, S. J., and Hogan, M. E. (1988) *Science* **241,** 456–459.
6. Maher, L. J. I., Wold, B., and Dervan, P. B. (1989) *Science* **245,** 725–730.
7. Okada, T., Yamaguchi, K., and Yamashita, J. (1994) *Growth Factors* **11,** 259–270.
8. Ekstrand, A. J., James, C. D., Cavenee, W. K., and Collins, V. P. (1991) *Cancer Res.* **51,** 2164–2172.
9. Libermann, T. A., Nusbaum, H. R., and Razon, N. (1985) *Nature* **313,** 144–147.
10. Han, H., and Dervan, P. B. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3806–3810.
11. Roberts, R. W., and Crothers, D. M. (1992) *Science* **258,** 1463–1466.
12. Short, M. P., Choi, B. C., Lee, J. K., Malick, A., Breakefield, X. O., and Martuza, R. L. (1990) *J. Neurosci. Res.* **27,** 427–433.
13. Ezzeddine, Z. D., Martuza, R. L., and Platika, D. (1991) *New Biol.* **3,** 608–614.
14. Culver, K. W., Ram, Z., and Wallbridge, S. (1992) *Science* **256,** 1550–1552.
15. Takamiya, Y., Short, M. P., Ezzeddine, Z. D., Moolten, F. L., Breakefield, X. O., and Martuza, R. L. (1992) *J. Neurosci. Res.* **33,** 493–503.
16. Takamiya, Y., Short, M. P., Moolten, F. L., Fleet, C., Mineta, R., Breakefield, X. O., and Martuza, R. L. (1993) *J. Neurosurg.* **79,** 104–110.
17. Yamada, M., Shimizu, K., Miyao, Y., Hayakawa, T., Ikenaka, K., Nakahira, K., Nakajima, K., Kagawa, T., and Mikoshiba, K. (1992) *Jpn. J. Cancer Res.* **83,** 1244–1247.
18. Oldfield, E. H., Ram, Z., Culver, K. W., Blaese, R. M., Devroom, H. L., and Anderson, W. F. (1993) *Hum. Gene Ther.* **4,** 39–69.
19. Ram, Z., Culver, K. W., Walbridge, S., Blaese, R. M., and Oldfield, E. H. (1993) *Cancer Res.* **53,** 83–88.
20. Miyao, Y., Shimizu, K., Moriuchi, S., Yamada, M., Nakahira, K., Nakajima, K., Nakao, J., Kuriyama, S., Tsuji, T., Mikoshiba, K., Hayakawa, T., and Ikenaka K. (1993) *J. Neurosci. Res.* **36,** 472–479.
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) In molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
22. Miller, A. D., and Rosman, G. J. (1989) *Bio Techniques* **7,** 980–990.
23. de Wet, J. R., Wood, K. V., Deluca, M., Helinski, D. R., and Subramani, S. (1987) *Mol. Cell. Biol.* **7,** 725–737.
24. Ishii, S., Xu, Y. H., Stratton, R. H., Roe, B. A., Merlino, G. T., and Pastan, I. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4920–4924.
25. Johnson, A. C., Ishii, S., Jinno, Y., Pastan, I., and Merlino, G. T. (1988) *J. Biol. Chem.* **263,** 5693–5699.
26. Danos, O., and Mulligan, R. C. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6460–6464.
27. Wigler, M., Pellicer, A., Silverstein, S., and Axel, R. (1978) *Cell* **14,** 725–731.
28. Cepko, C. (1989) *Methods Neurosci.* **1,** 367–392.
29. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
30. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–254.