Reduction of EGF receptor synthesis by antisense RNA vectors

John Hunts*, Glenn Merlino, Ira Pastan and Nobuyoshi Shimizu*+

*Dept of Molecular and Cellular Biology, University of Arizona, Tucson, AZ, USA, Laboratory of Molecular Biology, NCI, Bethesda, MD, USA and +Dept of Molecular Biology, Keio University School of Medicine, Tokyo, Japan

Received 30 July 1986

An association has been observed between squamous cell carcinomas and elevated EGF receptor levels. The approach taken here to analyze this problem was to construct vectors which express antisense RNA for the EGF receptor. The plasmid vector used was derived from pSV2-neo, which is readily selectable. Three EGF receptor cDNA fragments, a 5'-, a middle and a 3'-region were tested for differences in efficiency as antisense RNA. The results suggest that the 5'-fragment was the most efficient.

antisense RNA *EGF receptor* *(Squamous cell carcinoma)*

1. INTRODUCTION

The EGF receptor (EGFR) gene has been implicated as a proto-oncogene by both qualitative [1] and quantitative evidence [2–4]. One technique which may prove useful in studying EGFR function and activity is the use of complementary or antisense RNA. Antisense RNA has been used with some success for the thymidine kinase (TK) gene and an actin gene [5,6]. By reversing the direction of the gene of interest relative to its promoter, the opposite strand of DNA will be transcribed, generating a complementary antisense RNA. This antisense RNA can then hybridize with the sense RNA to inhibit its translation into protein. In the case of the antisense RNA which is generated in the nucleus, the hybridization is thought to inhibit gene expression by preventing RNA transport out of the nucleus [6].

Elevated EGFR levels have been associated with squamous cell carcinomas [2–4]. Furthermore, at least 3 mechanisms have the potential to increase the level of EGFR on the cell surface [7]. To understand better what role(s) elevated EGFR levels play in the transformation process, antisense EGFR-RNA vectors were tested for efficiency of EGFR inhibition. Three different fragments of the EGFR cDNA were tested for antisense RNA, a 5'-, a middle and a 3'-fragment. The 5'-fragment was observed to be the most efficient in reducing EGFR levels in a squamous cell carcinoma cell line.

2. MATERIALS AND METHODS

2.1. *Recombinant DNA*

Techniques and procedures were performed as described [8]. The parent plasmid used was pSV2neo [9], which allows resistance to the antibiotic G-418 (Gibco).

2.2. *Cell culture*

The squamous cell carcinoma cell line NA [10] was maintained at 37°C in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin in the presence of 10% CO_2.

2.3. *DNA transfection*

The vector DNA was introduced into NA cells

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

by calcium phosphate-DNA co-precipitation [11]. Briefly, 5×10^5 cells plated the previous night in 90 mm dishes were exposed to precipitated DNA (20 μg/plate) for 24 h at 37°C. The next day the medium was changed and after 48 h of the initial DNA exposure, the cells were placed under G-418 selection. Single colonies appeared approx. 14 days later and were isolated with cloning rings.

2.4. *EGF binding*

Confluent cells in 24 well dishes (2 cm^2) were incubated in ice (0°C) for 2 h in the presence of 100 ng/ml ^{125}I-EGF (plus 1 μg/ml unlabeled EGF for non-specific binding) in a total volume of 0.2 ml of EBSS, pH 7.4, 5 mM Hepes and 1 mg/ml BSA.

3. RESULTS

Initially, a general purpose vector was constructed using the pSV2neo plasmid [9]. The pSV2neo plasmid was chosen because it has a selectable marker, neo, which gives cells resistance to the drug G-418. Fig.1 shows the outline of the construction of the expression vector pEN. A *Hin*dIII site was first deleted from pSV2neo. This change did not disrupt the neo gene activity. An *Eco*RI-*Bam*HI 375 bp fragment from pBR322 was then added to this plasmid. An SV40 poly(A) site from pMTVdhfr [12] was put into the filled *Eco*RI site, generating pEN. The pEN vector has 3 unique restriction sites *Cla*I, *Hin*dIII, and *Bam*HI.

The vector pEN was then used to construct antisense-EGFR vectors. Fig.2a shows the vectors which were constructed. All the vectors contain the AEV LTR as a promoter [13] inserted in the *Hin*dIII site of pEN. Three fragments from the EGFR cDNA were chosen (fig.2a). A 717 bp *Pvu*II-*Cla*I 5′-fragment was used [14] and filled in and inserted in the filled *Cla*I site of pEN, generating pERR5. Likewise, a middle *Bam*HI fragment of 1032 bp was isolated [14] and filled in and inserted in pEN at the filled *Cla*I site, giving rise to pERRM. Similarly, a 3′-*Cla*I fragment of 1076 bp was isolated [14] and inserted in the *Cla*I site of pEN, giving pERR3. All EGFR inserts were selected for in the opposite orientation to the promoter relative to the normal gene (fig.2b).

The vectors were introduced into NA cells, EGFR hyperproducers, by calcium phosphate-

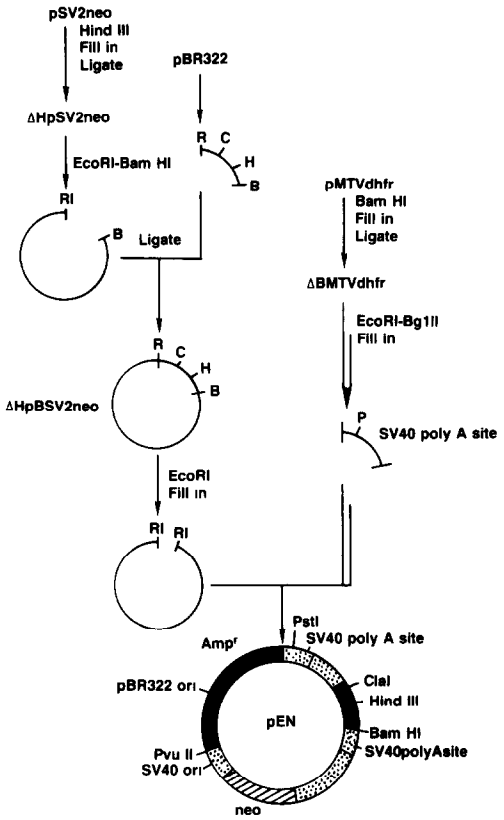

Fig.1. Construction of pEN. pSV2neo [9] was modified with pBR322 to contain 3 unique restriction sites. The *Eco*RI-*Bgl*II fragment from pMTVdhfr [11] contains the polyadenylation site and intron for the small T antigen of SV40. Restriction enzymes are: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I. The dark regions are derived from pBR322, the stippled regions from SV40 and the hatched box is the neo gene.

DNA precipitation [11]. Cells possessing the integrated vector were then selected for by using the drug G-418 [9]. Single colonies were isolated using cloning rings. These G-418 resistant colonies were then checked for EGF binding relative to the parental NA cells. The results of the binding assays from several different clones selected from pERR5, pERRM and pERR3 are shown in fig.3. The results indicate that pERR5, containing the 5′-end of the EGFR cDNA was the most efficient at inhibiting EGFR expression. A decrease of up to 52% was seen in pERR5 clone 5-1 (fig.3a). The average for the pERR5 clones was a reduction of EGF binding by 26% (fig.3b). The other vectors

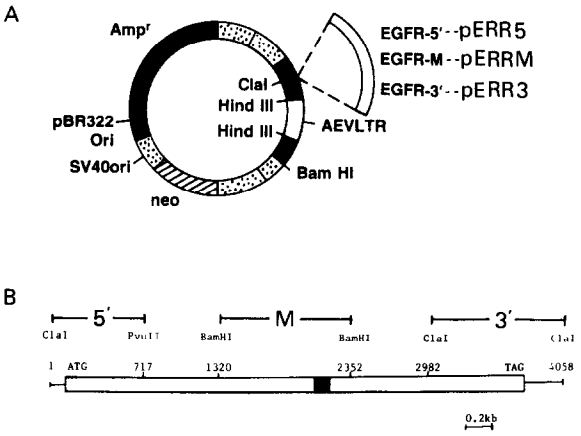

Fig.2. Antisense EGFR-RNA vectors. (A) Three antisense vectors were constructed with the EGFR cDNA fragment oriented in the opposite direction as to the retroviral AEVLTR promoter. The names of the vectors were pERR5, pERRM and pERR3, which contain 5'-, M and 3'-fragments, respectively. (B) EGFR cDNA fragments used for each of the vectors with the dark region showing the transmembrane domain (for a complete description of EGFR cDNAs see [14]).

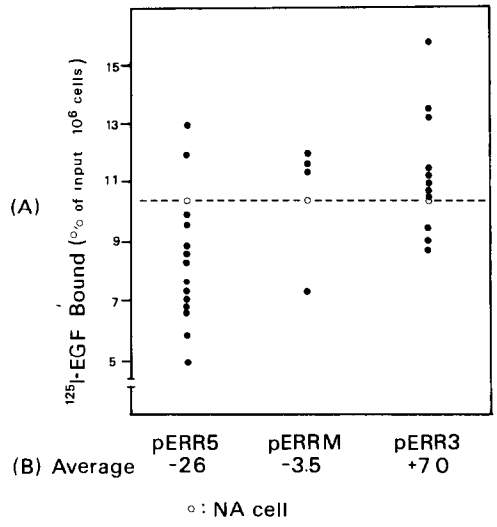

Fig.3. EGF binding. (A) Clones isolated using each of the three vectors were tested for EGF binding relative to the parental cell line NA (NA binding shown as horizontal line). (B) The average percent change in EGF binding for the three vectors relative to NA.

pERRM and pERR3 showed little or no reduction in EGF binding. These results suggest that the 5'-fragment of the EGFR cDNA is the most efficient for antiserum-RNA inhibition of EGFR gene expression in NA.

4. DISCUSSION

The squamous cell carcinoma cell line used for testing these vectors, NA, has a 20–30-fold EGFR gene amplification [10] accompanied by a similar high level of EGFR mRNA [10]. This suggests that in order for the EGFR levels to be reduced more in NA, a larger amount of EGFR antisense RNA must be produced. We are presently testing an amplification vector [6] for this. The antisense RNA is linked to the mRNA for the dihydrofolate reductase (dhfr) gene by inserting the antisense gene between dhfr and its poly(A) site, generating a bi-functional mRNA. Reducing the EGFR levels in NA could change some properties of NA and give more information as to what effect high EGFR levels have in these transformed cells, such as changing the growth characteristics. Similarly, if the endogenous EGFR gene expression could be reduced to zero, rearranged or altered EGFR genes could then be introduced to characterize better the receptor function.

ACKNOWLEDGEMENTS

We thank Professor K. Toyoshima and Dr T. Yamamoto for the AEV LTR.

REFERENCES

[1] Downward, J., Yarden, Y., Mayes, E., Scarce, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M.D. (1984) Nature 307, 521–527.

[2] Cowley, G., Smith, J.A., Gusterson, B., Hendler, F. and Ozanne, B. (1984) in: Cancer Cells (Levine, A. et al. eds) pp.5–10, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

[3] Libermann, T.A., Nusbaum, H.R., Razon, N., Kris, R., Lax, I., Soreq, H., Wittle, N., Waterfield, M.D., Ullrich, A. and Schlessinger, J. (1985) Nature 313, 144–147.

[4] Hunts, J., Ueda, M., Ozawa, S., Abe, O., Pastan, I. and Shimizu, N. (1985) J. Cancer Res. 76, 663–666.

[5] Izant, J.G. and Weintraub, H. (1985) Science 229, 345–352.
[6] Kim, S.K. and Wold, B.J. (1985) Cell 42, 129–138.
[7] Hunts, J., Gamou, S., Hirai, M. and Shimizu, N. (1986) Jap. J. Cancer Res., in press.
[8] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.
[9] Southern, P.J. and Berg, P. (1982) J. Mol. Appl. Genet. 1, 337–341.
[10] Yamamoto, T., Kamata, N., Kawano, H., Shimizu, S., Kuroki, T., Toyoshima, K., Rikimaru, K., Nomura, N., Ishizaki, R., Pastan, I., Gamou, S. and Shimizu, N. (1986) Cancer Res. 46, 414–416.
[11] Corsaro, C.M. and Pearson, M.L. (1981) Som. Cell Genet. 7, 603–616.
[12] Lee, F., Mulligan, R., Berg, P. and Ringold, G. (1981) Nature 294, 228–232.
[13] Yamamoto, T., Hihara, H., Nishida, T., Kawai, S. and Toyoshima, K. (1983) Cell 34, 225–232.
[14] Merlino, G.T., Ishii, S., Whang-Peng, J., Knutsen, T., Xu, Y., Clark, A.J.L., Stratton, R.A., Wilson, R.K., Ma, D.P., Roe, B.A., Hunts, J.H., Shimizu, N. and Pastan, I. (1985) Mol. Cell. Biol. 5, 1722–1734.