Animals that eat or drink in response to ESLH are much more likely to display SIP. The finding that all ESLH-neg animals that drank water during the SIP testing sessions became ESLH-pos suggests that a tendency to display ingestive behavior when active may transfer from one situation to another.

A great many nonspecific stimuli, such as noise, social facilitation, and tail pinch have been reported to initiate eating in animals or humans (*17, 18*). What seems to be common to all these stimuli is that they cause animals to become more active. When hungry animals are given small amounts of food at regular intervals they become highly active and possibly more responsive to environmental stimuli. Several investigators have observed that animals from a variety of species tend to display attack or escape behaviors during SIP sessions (*10*). We observed that during the SIP testing animals frequently jumped vigorously toward the top of the chamber in an apparent attempt to escape, and at the end of the session they were often difficult to handle (on one occasion biting the experimenter). Although animals do not normally become difficult to handle after ESLH, they are consistently highly active during the stimulation.

What needs to be explained is why some animals more readily direct this increased activity into eating and drinking. Any answer must take into consideration the fact that the response to ESLH is not correlated with unrestricted food and water consumption and presumably not with differences in hunger or thirst states that are regulated by nutritional needs. To our knowledge, this investigation represents the first attempt to study the consistency of individual differences in readiness to ingest food or water in response to different arousing stimuli. The results suggest that this approach may help in searching for underlying mechanisms by more fully describing the behavior to be explained.

GUY MITTLEMAN
ELLIOT S. VALENSTEIN
Department of Psychology,
and *Neuroscience Laboratory,*
University of Michigan,
Ann Arbor 48109

References and Notes

1. E. S. Valenstein, *Brain Behav. Evol.* **2**, 295 (1969).
2. ———, V. C. Cox, J. W. Kakolewski, *Psychol. Rev.* **77**, 16 (1970).
3. M. Devor, R. A. Wise, N. W. Milgram, B. G. Hoebel, *J. Comp. Physiol. Psychol.* **73**, 226 (1970); E. E. Coons, M. Levak, N. E. Miller, *Science* **150**, 1320 (1965).
4. R. A. Wise, *Brain Res.* **67**, 187 (1974); E. S. Valenstein, in *Nebraska Symposium on Motivation 1974*, J. K. Cole and T. B. Sonderegger, Eds. (Univ. of Nebraska Press, Lincoln, 1975), p. 251.
5. E. S. Valenstein, in *Neurosurgical Treatment in Psychiatry, Pain, and Epilepsy*, W. H. Sweet, S. Obrador, J. Martin-Rodriguez, Eds. (University Park Press, Baltimore, 1977), p. 27.
6. R. A. Wise, *Physiol. Behav.* **6**, 569 (1971).
7. S. E. Bachus and E. S. Valenstein, *ibid.* **23**, 421 (1979).
8. G. Mittleman and E. S. Valenstein, *ibid.* **26**, 371 (1981).
9. J. L. Falk, *Science* **133**, 195 (1961).
10. ———, *Physiol. Behav.* **6**, 577 (1971).
11. D. Levitsky and G. Collier, *ibid.* **22**, 223 (1968); E. S. Valenstein, *J. Psychiatr. Res.* **8**, 335 (1971); L. D. Devenport, *J. Comp. Physiol. Psychol.* **92**, 651 (1978).
12. T. W. Robbins and G. F. Koob, *Nature (London)* **285**, 409 (1980); A. G. Phillips and R. S. Nikaido, *ibid.* **258**, 750 (1975).
13. M. Wallace, G. Singer, J. Finlay, S. Gibson, *Pharmacol. Biochem. Behav.* **18**, 129 (1983); L. P. Brett and S. Levine, *J. Comp. Physiol. Psychol.* **93**, 946 (1979).
14. M. J. Wayner, F. C. Barone, C. C. Loullis, in *Handbook of the Hypothalamus*, P. J. Morgane and J. Panksepp, Eds. (Dekker, New York, 1981), p. 107.
15. Stereotaxic coordinates: skull level between lambda and bregma; 3.50 mm posterior to bregma, 1.50 mm lateral to the midline, and 8.40 mm below the dorsal surface of the skull.
16. Data were analyzed by a two-factor analysis of variance for repeated measures. The ESLH-pos rats consumed significantly more water than did ESLH-neg rats during each of the ten SIP sessions [$F(1, 31) = 5.6$, $P < 0.025$] and increased the amount consumed over sessions at a significantly greater rate [$F(9, 279) = 3.18$, $P < 0.001$]. Similarly, the percentage of ESLH-pos animals that drank was significantly greater on each session [$F(1, 31) = 15.9$, $P < 0.001$].
17. T. W. Robbins and P. J. Fray, *Appetite* **1**, 103 (1980).
18. E. S. Valenstein *et al.*, *Behav. Neural Biol.* **34**, 271 (1982). This study demonstrated strain differences in readiness to eat in response to tail-pinch.
19. We thank D. M. Camp, T. E. Robinson, and M. Lewis for helpful comments on the manuscript, and B. J. Martonyi for technical assistance.

8 November 1983; accepted 15 February 1984

Amplification and Enhanced Expression of the Epidermal Growth Factor Receptor Gene in A431 Human Carcinoma Cells

Abstract. *The sequence of the human epidermal growth factor (EGF) receptor shows great homology with the avian erythroblastosis virus v-*erb *B oncogene, raising the possibility that the receptor gene is identical to the c-*erb *B protooncogene. Human A431 epidermoid carcinoma cells, which have an unusually high number of EGF receptors, were examined to determine whether elevated EGF receptor levels correlate with gene amplification. Southern blots of genomic DNA's from A431 and other human cell lines were probed with either a v-*erb *B gene fragment or a human EGF receptor complementary DNA clone (pE7), previously isolated from an A431 complementary DNA library. When either probe was used to analyze Eco RI– or Hind III–generated DNA fragments, EGF receptor DNA sequences were amplified about 30-fold in A431. Differences in the banding pattern of A431 DNA fragments relative to normal fibroblast DNA indicate the occurrence of a rearrangement in the region of the receptor gene. Furthermore, A431 cells contain a characteristic, prominent 2.9-kilobase RNA. These results are consistent with the hypothesis that, in A431 cells, gene amplification, possibly associated with a translocation event, may result in the overproduction of EGF receptor protein or the appearance of the transformed phenotype (or both).*

Many retroviruses induce malignant transformation in cells via expression of specific proviral DNA sequences (v-onc). Mammalian cells also contain evolutionarily conserved homologous counterparts to these transforming genes (c-onc) (*1, 2*). It has been proposed that enhanced expression of cellular oncogenes may result in the manifestation of the malignant phenotype (*3–5*).

One retrovirus, avian erythroblastosis virus (AEV), induces both erythroblastosis and sarcomas in infected chickens (*6, 7*). The AEV is replication defective; portions of the replication genes are replaced by a region implicated in cellular oncogenesis (*erb*) (*8–11*). The AEV *erb* region consists of two putative oncogenes, A and B, whose human cellular homologs (c-*erb*) are located on separate chromosomes (*12, 13*). The main protein product of the v-*erb* B gene is approximately 62,000 daltons before modification (*14*) and is the cause of erythroblastosis and sarcomas (*15, 16*).

Recently, the amino acid sequence of six distinct peptides from the human epidermal growth factor (EGF) receptor have been shown to be strikingly similar to that of the v-*erb* B transforming protein (*17*). It is therefore possible that the EGF receptor and the c-*erb* B gene product are derived from the same cellular gene.

The human epidermoid carcinoma cell line A431 has an unusually high number of EGF receptors (approximately 3×10^6 sites per cell) (*18, 19*) when compared with normal human fibroblasts and other cell types (about 1×10^5 sites per cell) (*20*). This has been shown by measuring the number of binding sites for ^{125}I-labeled EGF or by immunoprecipitation of the receptor from [^{35}S]meth-

ionine-labeled cells with receptor-specific antibodies (*21*). We took advantage of the similarity between the EGF receptor and the v-*erb* B gene product (*17*) to determine whether high levels of EGF receptor in A431 cancer cells are correlated with receptor gene copy number.

Initially, the conserved "*src* family" domain (0.5-kbp Bam HI fragment) of the v-*erb* B gene (*22, 23*) was nick-translated and used as a probe to hybridize to nitrocellulose blots of cleaved human genomic DNA from several cell lines. The v-*erb* B probe, when used at relatively low stringency [5× SSC (standard saline citrate), 63°C], hybridizes to several Eco RI and Hind III A431 DNA fragments (lane 3 in Fig. 1, a and b) whose signal appears to be amplified relative to other transformed cell lines expressing much lower levels of EGF receptor: human leukemic lymphoblast CEM and epidermoid carcinoma KB (lanes 1 and 2 in Fig. 1, a and b). A probe for the human α1(I) collagen gene which is represented as a single copy in the human genome showed no basic difference between cell types when hybridized to this same blot (data not shown). The finding that a gene coding for human c-*erb* B protein is amplified in certain cultured cells agrees with the reported amplification of other oncogenes, most notably for c-*myc* and c-Ki-*ras* (*24–28*).

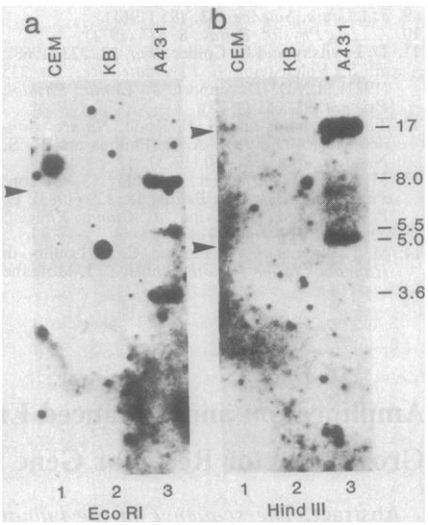
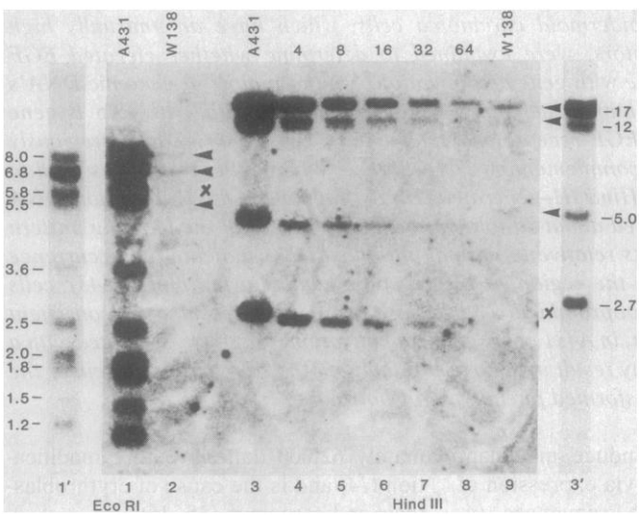


Fig. 1. Hybridization of the v-*erb* B DNA probe to cellular genomic DNA. High molecular weight genomic DNA was isolated from cells in culture by sodium dodecyl sulfate–proteinase K solubilization, phenol-chloroform extraction [modified from (*39*)], and treatment with ethanol. Cellular DNA was then digested with Eco RI (a) or Hind III (b), electrophoretically fractionated on 1 percent agarose, transferred to nitrocellulose paper (*40*), and hybridized to a ^{32}P-labeled, nick-translated (*41*), 0.5-kbp Bam HI v-*erb* B fragment. Hybridization and washing procedures were as described (*42*) except that cross-hybridization was performed at 63°C. (Lane 1) Fifteen micrograms of DNA from CEM cells; (lane 2) 10 μg of DNA from KB cells; (lane 3) 15 μg of DNA from A431 cells. Arrowheads indicate the location of clearly detectable DNA bands in nonamplified c-*erb* B genes. Sizes are in kilobase pairs.



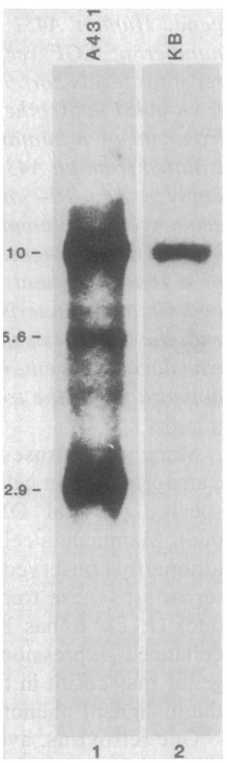


Fig. 2 (left). Hybridization of the human EGF receptor clone (pE7) to either Eco RI (lanes 1 and 2) or Hind III (lanes 3 to 9) digested genomic DNA. (Lanes 1 and 3) A431 DNA; (lanes 2 and 9) WI38 human embryo fibroblast DNA; (lanes 1′ and 3′) lighter exposures of lanes 1 and 3. Blots containing 15 μg of each DNA per well were prepared as in Fig. 1, then hybridized to the nick-translated 2.3-kbp insert of pE7 in 50 percent formamide, 5× SSC, 42°C, and then washed stringently (0.2× SSC, 60°C) (*43*). Arrowheads indicate the location of detectable DNA bands in cells containing nonamplified genes. A cross marks the position of DNA fragments in the amplified A431 gene but undetectable in other cellular DNA's. Serial dilutions of Hind III–restricted A431 DNA were performed, with the addition of appropriate amounts of sheared salmon sperm DNA to each sample to achieve equivalent quantities of genomic DNA on each well. (Lane 4) 1:4 dilution; (lane 5) 1:8; (lane 6) 1:16; (lane 7) 1:32; (lane 8) 1:64. The salt in the added salmon sperm DNA caused the movement of the DNA fragments in lanes 4 to 8 to be slightly altered. Sizes are in kilobase pairs. Fig. 3 (right). EGF receptor–specific RNA's in A431 cells and KB cells. Total RNA was isolated by guanidine isothiocyanate solubilization and cesium chloride centrifugation (*44*), and then subjected to oligo(dT)-cellulose affinity chromatography (two successive treatments). Polyadenylated RNA (5 μg per well) was fractionated on 1 percent agarose-formaldehyde (*46*), transferred to nitrocellulose (*46*), and hybridized to nick-translated pE7 DNA insert. (Lane 1) A431 polyadenylated RNA; (lane 2) KB polyadenylated RNA. Sizes are in kilobases. The original autoradiogram of lane 2 showed a faint band at 5.6 kb, but no band at 2.9, regardless of exposure time.

The fact that the v-*erb* B probe hybridizes to amplified sequences in a cell line expressing high levels of EGF receptor is consistent with the conclusion that, under these conditions, v-*erb* B hybridizes to the EGF receptor gene. However, these initial results were obtained by hybridizing an avian virus probe to human genomic DNA at relatively low stringency, making it difficult to be certain if any hybridizable DNA fragments actually represented EGF receptor gene-specific DNA. To circumvent this problem we isolated human complementary DNA (cDNA) clones from an A431 cDNA library cloned in pBR322 which could be hybridized at increased stringency and therefore higher specificity. These clones were isolated with the ^{32}P-labeled v-*erb* B 0.5-kbp Bam HI fragment as a probe. One of these (pE7) contains a 2.3-kbp insert that shows great homology to the published v-*erb* B sequences (*22, 23*), and complete homology with the published peptide sequences for the human EGF receptor protein (*17, 29*). Isolation of this clone allowed us to probe for EGF receptor gene sequences with confidence.

The 2.3-kbp insert of pE7 was isolated, nick-translated, and hybridized under more stringent conditions (5× SSC, 42°C, 50 percent formamide) to blots containing genomic DNA from A431 cells and diploid human embryo fibroblasts (WI38) restricted with either Eco RI or Hind III. The A431 DNA fragments that hybridize with the pE7 probe (lanes 1 and 3 in Fig. 2) give a much more intense signal than those from WI38 (lanes 2 and 9), indicating that the copy number of the EGF receptor gene sequences in A431 cells is much higher. A revertant A431 cell line (clone 1) (*30*) was also amplified, but the copy number was about half that of A431 (data not shown).

The level of amplification was estimated to be on the order of 30-fold by serial dilution of A431 genomic DNA (Fig. 2, compare lane 9 with lanes 7 and 8). Additional bands are present in this blot (compare Fig. 2, lane 1 with Fig. 1a, lane 3) because the insert contains a segment of ~ 1.7 kbp that hybridizes to the region of the EGF receptor gene 5′ to that recognized by the v-*erb* B probe. Most of the bands on the v-*erb* B blot are also present on the pE7 blot. The fact that a large number of bands totaling approximately 40 kbp are observed when Eco RI–digested genomic DNA is hybridized to a smaller 2.3-kbp cloned cDNA insert suggests that either the gene is very large with many introns, or that the probe picks up amplified EGF receptor gene sequences at more than one site. Further study is required to resolve this question.

The c-*erb* B gene and the EGF receptor gene have been shown to map to overlapping regions of chromosome 7 (*13, 31*). This finding further supports the idea that the two genes are the same. A431 cells have recently been reported to be hypotetraploid, with two copies of intact chromosome 7, and two types of translocation chromosomes involving chromosome 7 (*32*). One of the translocated chromosomes (M4) was found to be responsible for high EGF binding ability when introduced into mouse A9 cells by somatic cell hybridization (*32*). Because gene amplification occurs at sites of chromosome rearrangement, as has been demonstrated in methotrexate-resistant cells (*33, 34*), we looked more carefully at this possible correlation. Most of the larger, more prominent DNA fragments from A431 cells identified as EGF receptor–specific appeared to have counterparts of similar size in other cellular DNA's not exhibiting amplification (Figs. 1 and 2, arrowheads). However, at least two bands found in A431 genomic DNA were undetectable in WI38 DNA (Fig. 2, cross). This result suggests that a DNA rearrangement alters the genomic restriction pattern of the EGF receptor gene, an event that may be related to the gene amplification (*34*). An attractive hypothesis is that the human translocation chromosome M4 carries this DNA rearrangement.

Certain cell lines expressing high levels of either *myc* or *myb* protein exhibit both a translocation event and the appearance of an RNA species of unusual size (*27, 35–37*). Presumably, the translocation event alters the normal start site, stop site, or splicing pattern of the oncogene messenger RNA. Therefore, we compared polyadenylated RNA from A431 cells and KB cells to determine whether new species of RNA were detectable. Polyadenylated RNA was electrophoretically fractionated on 1 percent agarose, transferred to nitrocellulose, and probed with the ^{32}P-labeled pE7 insert. The results (Fig. 3) reveal that two RNA species of about 10 kb and 5.6 kb were present in both cell types. However, in A431 cells a new band of 2.9 kb appeared. This result is consistent with the hypothesis that in A431 a modified RNA species is transcribed from a rearranged and possibly translocated chromosome. Since there are two reported translocations involving chromosome 7 in A431 (*32*), this 2.9-kb species could conceivably come from either of the rearranged chromosomes. The 10-kb and 5.6-kb species were also elevated in A431 cells relative to KB cells. It is possible that several different levels of regulation are responsible for the enhanced accumulation of these different RNA species.

We have demonstrated that EGF receptor gene sequences are amplified in A431 cells. Because A431 cells have a marker chromosome (M4) which is associated with a translocation event, and is responsible for increased production of EGF receptor in hybrids of human and mouse cells (*32*), it is possible that gene amplification or chromosomal translocation (or both) in A431 cells result in overproduction of modified EGF receptor (c-*erb* B) RNA and its corresponding protein. The enhanced expression of this gene could also be related to the appearance of the transformed phenotype in the A431 cell line, as has been sugeted for overproduction of oncogene products in other cell types (*24–27, 38*).

GLENN T. MERLINO
YOUNG-HUA XU
SHUNSUKE ISHII
ADRIAN J. L. CLARK
Laboratory of Molecular Biology, National Cancer Institute, Bethesda, Maryland 20205
KENTARO SEMBA
KUMAO TOYOSHIMA
TADASHI YAMAMOTO
Institute of Medical Science, University of Tokyo, Japan
IRA PASTAN
Laboratory of Molecular Biology, National Cancer Institute

References and Notes

1. P. M. Duesberg, *Cold Spring Harbor Symp. Quant. Biol.* **44**, 13 (1980).
2. J. M. Bishop, *Cell* **23**, 5 (1981).
3. W. S. Hayward, B. G. Neel, S. M. Astrin, *Nature (London)* **290**, 475 (1981).
4. G. Klein, *ibid.* **294**, 313 (1981).
5. J. M. Bishop, *Annu. Rev. Biochem.* **52**, 301 (1983).
6. J. Engelbreth-Holm and A. Meyer Rothe, *Acta Pathol. Microbiol. Scand.* **12**, 352 (1935).
7. T. Graf, B. Royer-Pokora, G. E. Schubert, H. Beug, *Virology* **71**, 423 (1976).
8. K. Bister and P. M. Duesberg, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5023 (1979).
9. M. Roussel, S. Saule, C. Lagron, C. Rommens, H. Beug, T. Graf, D. Stehelin, *Nature (London)* **282**, 452 (1979).
10. M. M. C. Lai, S. S. F. Hu, P. K. Vogt, *Virology* **97**, 366 (1979).
11. B. Vennström, L. Fanshier, C. Moscovici, J. M. Bishop, *J. Virol.* **36**, 575 (1980).
12. B. Vennström and J. M. Bishop, *Cell* **28**, 135 (1982).
13. N. K. Spurr, E. Solomon, M. Jansson, D. Sheer, P. N. Goodfellow, W. F. Bodmer, B. Vennström, *EMBO J.* **3**, 159 (1984).
14. M. L. Privalsky and J. M. Bishop, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3958 (1982).
15. L. Frykberg, S. Palmieri, H. Beug, T. Graf, M. J. Hayman, B. Vennström, *Cell* **32**, 227 (1983).
16. T. Yamamoto, H. Hihara, T. Nishida, S. Kawai, K. Toyoshima, *ibid.* **34**, 225 (1983).
17. J. Downward, Y. Yarden, E. Mayes, G. Scrace, N. Totty, P. Stockwell, A. Ullrich, J. Schlessinger, M. D. Waterfield, *Nature (London)* **307**, 521 (1984).
18. R. N. Fabricant, J. E. DeLarco, G. J. Todaro, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 565 (1977).
19. H. Haigler, J. F. Ash, S. J. Singer, S. Cohen, *ibid.* **75**, 3317 (1978).
20. G. Carpenter, K. J. Lemback, M. M. Morrison, S. Cohen, *J. Biol. Chem.* **250**, 4297 (1975).
21. C. M. Stoscheck and G. Carpenter, *J. Cell Biol.* **98**, 1048 (1984); N. Richert and I. Pastan, unpublished data.
22. T. Yamamoto, T. Nishida, N. Miyajima, S. Kawai, T. Ooi, K. Toyoshima, *Cell* **35**, 71 (1983).
23. M. L. Privalsky, R. Ralston, J. M. Bishop, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 704 (1984); see also (*22*).
24. S. Collins and M. Groudine, *Nature (London)* **298**, 679 (1982).
25. R. Dalla-Favera, F. Wong-Staal, R. C. Gallo, *ibid.* **299**, 61 (1982).
26. K. Alitilo, M. Schwab, C. C. Liu, H. E. Varmus, J. M. Bishop, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1707 (1983).
27. M. Schwab, K. Alitalo, H. E. Varmus, J. M. Bishop, D. George, *Nature (London)* **303**, 497 (1983).
28. M. Schwab, K. Alitalo, K. H. Klempnauer, H. Varmus, J. M. Bishop, F. Gilbert, G. Brodeur, M. Goldstein, J. Trant, *ibid.* **305**, 245 (1983).
29. Y.-H. Xu, S. Ishii, A. J. L. Clark, B. Roe, D. P. Ma, R. K. Wilson, G. Merlino, I. Pastan, in preparation.
30. A. Lifshitz, C. S. Lazar, J. E. Buss, G. N. Gill, *J. Cell. Physiol.* **115**, 235 (1983).
31. I. Kondo and N. Shimizu, *Cytogenet. Cell Genet.* **35**, 9 (1983).
32. N. Shimizu, I. Kondo, S. Gamou, M. A. Behzedian, Y. Shimizu, *Som. Cell Mol. Genet.* **10**, 45 (1984).
33. F. W. Alt, R. E. Kellems, J. R. Bertino, R. T. Schimke, *J. Biol. Chem.* **253**, 1357 (1978).
34. W. F. Flintoff, E. Livingston, C. Duff, R. G. Worton, *Mol. Cell. Biol.* **4**, 69 (1984).
35. J. F. Mushinski, S. R. Bauer, M. Potter, E. P. Reddy, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1073 (1983).
36. J. F. Mushinski, M. Potter, S. R. Bauer, E. P. Reddy, *Science* **220**, 795 (1983).
37. K. B. Marcu, L. J. Harris, L. W. Stanton, J. Erikson, R. Watt, C. M. Croce, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 519 (1983).
38. Y.-K. T. Fung, W. G. Lewis, L. B. Crittenden, H.-J. Kung, *Cell* **33**, 357 (1983).
39. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1982).
40. E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975).
41. T. Maniatis, S. G. Kee, A. Efstratiadis, F. C. Kafatos, *Cell* **8**, 163 (1976).
42. P. A. Overbeek, G. T. Merlino, N. K. Peters, V. H. Cohn, G. P. Moore, L. J. Kleinsmith, *Biochim. Biophys. Acta* **656**, 195 (1981).
43. C. McKeon, H. Ohkubo, I. Pastan, B. de Crombrugghe, *Cell* **29**, 203 (1982).
44. J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979).
45. G. T. Merlino, C. McKeon, B. de Crombrugghe, I. Pastan, *J. Biol. Chem.* **258**, 10041 (1983).
46. P. S. Thomas, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5201 (1980).
47. We thank Catherine McKeon, Maria Mudryj, Nancy Richert, Raji Padmanabhan, Bruce Howard, Benoit de Crombrugghe, Michael Gottesman for advice and aid in the completion of this work. We thank Len Neckers for CEM cells.

30 March 1984; accepted 5 April 1984