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Cloning of a leucine-zipper protein recognized by the sera of patients with antibody-associated paraneoplastic cerebellar degeneration

(neurological syndrome/remote effects of cancer/tumor antigens)

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ABSTRACT **Antibody-associated paraneoplastic cerebellar degeneration (the Yo syndrome) is an uncommon disorder in which an immune response is specifically directed against tumor tissue and the cerebellum. Screening of a λ expression library has resulted in the isolation of cDNA clones that encode the major antigen recognized by serum from these patients. The fusion protein produced by the cDNA clones provides the basis of a simple diagnostic assay for this neurological syndrome. The occurrence of leucine-zipper and zinc-finger motifs in the predicted open reading frame suggests that this protein plays a role in the regulation of gene expression.**

Paraneoplastic cerebellar degeneration is a disorder of the cerebellum found in association with neoplasms of lung, ovary, breast, or Hodgkin disease (1). Neuropathological analysis of affected brains has revealed extensive loss of Purkinje cells, variable loss of granule and basket neurons, and proliferation of Bergman glia (2). The relationship between the primary tumor and the resultant cerebellar dysfunction is not clearly understood. The presence of infiltrating lymphocytes in some of the affected brains has suggested an immune mechanism (3).

A clinically definable subset of patients with paraneoplastic cerebellar degeneration harbor a characteristic antibody that has been called anti-Yo (4). Sera from these patients react with antigens expressed in the Purkinje cells of the normal cerebellum and in the tumor tissue of affected individuals (5). There is also evidence of increased antibody synthesis in the affected brains (6). These observations suggest a model for the neurological dysfunction in which an immune response primarily directed against a tumor antigen is misdirected against similar antigens peculiar to the cerebellum. On Western blot analysis of Purkinje cells and tumor tissue, the anti-Yo sera react with at least two antigens, a major species of 62 kDa (CDR-62) and a minor species of 34 kDa (CDR-34) (where CDR is cerebellar degeneration related) (7). The gene encoding the minor antigen (CDR-34) has been isolated and characterized (8, 9). We now report the isolation of cDNAs that encode the major Yo paraneoplastic antigen. Sequence analysis§ revealed CDR-62 to be a member of a family of the leucine-zipper DNA binding proteins. The availability of the recombinant protein has provided a simple diagnostic assay for the presence of anti-Yo antibodies.

MATERIALS AND METHODS

Sera from patients with antibody-associated paraneoplastic cerebellar degeneration was obtained from their physicians. A HeLa cell λ ZAP expression library was obtained from Stratagene.

Screening of λ HeLa Expression Library. Recombinant phage were screened at a density of 2×10^4 plaque-forming units per 150-mm plate of *Escherichia coli* XLI-Blue. After incubation for 6 hr at 37°C, the plates were overlaid with filters soaked in isopropyl β-D-thiogalactopyranoside (10 mM) and incubated for a further 12 hr at 37°C. The filters were then removed and incubated with anti-Yo sera (IgG, 2 μg/ml) for 2 hr at room temperature. The filters were then washed with TBST (50 mM Tris·HCl, pH 7.4/100 mM NaCl/0.2% Triton) and incubated with ^{125}I-labeled protein A (0.1 μCi/ml; 1 Ci = 37 GBq). After washing with TBST, the filters were exposed to Kodak XRA5 film at −70°C. Clones yielding positive signals were purified by several rounds of antibody screening until 100% of the plaques gave positive signals.

Analysis of Fusion Proteins. Phage clones were subcloned into Stratagene pBluescript (pBs) using the phage-rescue protocol (10). Individual clones were grown to an optical density of 0.6 at 600 nm and induced by adding isopropyl β-D-thiogalactopyranoside (10 mM). After 1 hr of induction at 37°C, the bacterial cells were isolated by centrifugation and resuspended in lysis buffer [2% (wt/vol) SDS/50 mM Tris·HCl, pH 6.8]. Lysates were then resolved by SDS/PAGE on 8% gels and transferred to nitrocellulose (11). The filters were then incubated with anti-Yo sera (5 μg/ml in TBST) for 2 hr at room temperature. The filters were then washed with TBST and incubated with ^{125}I-labeled protein A (0.1 μCi/ml). After a further washing with TBST, the filters were exposed to XRA5 film at −70°C.

Northern Blot Analysis. Total RNA was prepared from HeLa cells using guanidinium hydrochloride and a phenol/chloroform extraction method (10). The RNA was separated by agarose/formaldehyde gel electrophoresis in 1.2% gels and transferred to Hybond N (Amersham) according to the manufacturer's specification. The Hybond N filter was prehybridized with 50% (vol/vol) formamide/5× SSPE/0.05% polyvinylpyrrolidone/0.05% Ficoll/denatured DNA (200 μg/ml) (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA). The probe was synthesized from clone pY2 using T7 RNA polymerase and [α-^{32}P]UTP (10). The filter was hybridized with probe (1×10^7 cpm/ml) in prehybridization buffer at 55°C. The filter was then washed with 0.1× standard saline/citrate/0.1% SDS at 60°C and exposed to XRA5 film.

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Abbreviation: CDR, cerebellar degeneration related.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M63256).

DNA Sequence Analysis. All sequencing was based on the dideoxynucleotide chain-termination method (10). Double-stranded DNA was sequenced on both strands using SK, KS, M13 universal and reverse primers, and internal oligonucleotide primers. Sequences were merged and analyzed for open reading frame and functional motifs with the Macvector analysis software.

ELISA. Partially purified preparations of the fusion protein encoded by pY2 were adsorbed to 96-well microtiter plates. After blocking reactive sites with 2% (wt/vol) bovine serum albumin/phosphate-buffered saline (PBS), the wells were incubated with the appropriate concentration of sera (IgG, 2 μg/ml) diluted in 1% goat serum (2 hr at room temperature). Reactivity was determined by incubation with biotinylated goat anti-human IgG at a 1:1000 dilution followed by avidin–biotin-peroxidase complex. Peroxidase was measured by oxidation of *ortho*-phenyldiamine and absorbance was measured at 402 nm.

RESULTS

Isolation of Positive λ Clones. To clone the major Yo antigen (CDR-62), we have utilized our observation that this protein is abundantly expressed in the HeLa tumor cell line. Screening of the HeLa cell λZAP II expression library with a typical high-titer anti-Yo sera resulted in the isolation of two consistently positive clones (y1 and y2). None of these clones was recognized by normal human sera. The two clones were further analyzed by the epitope-selection method. In this procedure the antibodies that recognize the recombinant fusion protein were isolated and incubated with a Western blot of the tissue antigen. Purified antibodies from bona fide clones should identify the original antigen of interest. Fig. 1, lanes 2 and 3, shows that affinity-purified antibodies selected by reactivity with the fusion proteins encoded by clones y1 and y2 recognized CDR-62 expressed in human Purkinje neurons. A mock-purification of anti-Yo sera employing an irrelevant fusion protein served as the negative control (Fig. 1, lane 1). We concluded that both clones were related and encoded CDR-62.

Both λ phage clones were then subcloned into PBS utilizing the phagemid rescue procedure (10). The resulting bacterial plasmids pY1 and pY2 had inserts of 0.8 kilobase (kb) and 2.3 kb, respectively. Restriction enzyme digestion and hybridization analysis confirmed that the two clones were related and overlapped. A similar antibody screen was conducted

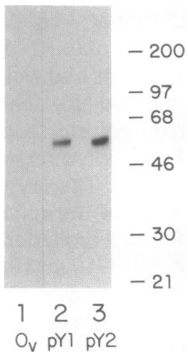

FIG. 1. Epitope selection analysis of pY1 and pY2. Nitrocellulose blots containing fusion proteins encoded by pY1, pY2, and as a control a β-galactosidase–ovalbumin fusion protein were incubated with anti-Yo sera (5 μg/ml) for 2 hr at room temperature. After washing with TBST, the IgG fraction bound to the filter was eluted with 0.1 M sodium citrate (pH 2.5). The purified IgG was then incubated with Western blot nitrocellulose strips containing protein from human Purkinje cells. Lanes: 1, negative control eluate from ovalbumin fusion protein; 2, eluate from pY1; 3, eluate from pY2. Molecular masses (in kDa) are shown.

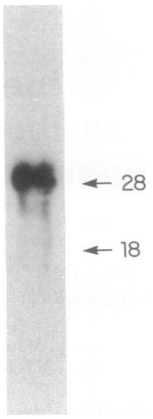

FIG. 2. Northern blot analysis of HeLa RNA. Total RNA was extracted from HeLa cells and separated by formaldehyde/agarose gel electrophoresis on 1.2% gels. The filter was incubated with a [^{32}P]RNA probe (1×10^7 cpm/ml), washed, and exposed to XRA5 film for 4 hr at −70°C. The arrows correspond to 18S (2.0 kb) and 28S (5.1 kb) rRNA.

using a human cerebellar expression library. Four clones were isolated and found to be related to pY1 and pY2 and were not analyzed further. Northern blot analysis of HeLa total RNA with probe synthesized from pY2 revealed an abundant transcript of 5.3 kb (Fig. 2).

Specific Recognition of cDNA Clones by Anti-Yo Sera. Preliminary experiments revealed that the fusion protein encoded by pY2 was the most reactive with anti-Yo sera. This clone was, therefore, the reagent of choice to establish a quantitative diagnostic assay. The reactivity of anti-Yo sera and various negative control sera was established by Western blot analysis of the fusion protein encoded by pY2. Anti-Yo sera identified a fusion protein of 55 kDa in extracts of pY2 (Fig. 3*A*, lane 1). No reactivity was observed with extracts of pBs (the parental plasmid vector with no insert (Fig. 3*A*, lane 1). The minor bands visible in Fig. 3*A*, lane 2, correspond to proteolytic fragments of the 55-kDa fusion protein. In view of this the fusion protein was routinely gel-purified and that

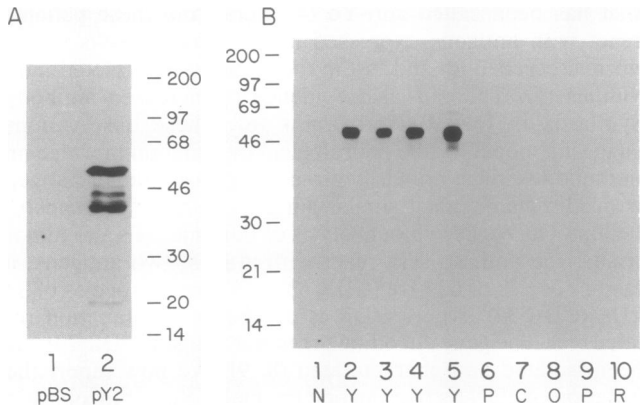

FIG. 3. Anti-Yo sera specifically react with the pY2 fusion protein. (*A*) Protein extracts (50 μg of total protein) from pBs (parental plasmid containing no insert) and pY2 were resolved by SDS/polyacrylamide gel electrophoresis in 10% gels, transferred to nitrocellulose, and incubated with anti-Yo sera (5 μg/ml; lanes 1 and 2, respectively). (*B*) The fusion protein encoded by pY2 was purified by preparative SDS/polyacrylamide gel electrophoresis on 10% gels. The partially purified protein was then electrophoresed on a "curtain well" SDS/polyacrylamide gel, transferred to nitrocellulose, and cut into strips of equal size. Strips (containing equal amounts of fusion protein) were then incubated with the following sera at 5 μg/ml. Lanes: 1, normal human sera; 2–5, anti-Yo positive sera; 6–10, anti-Yo negative sera. Molecular masses (in kDa) are shown.

purified material was used in Fig. 3*B*. Fig. 3*B* shows that sera previously characterized as anti-Yo were positive (lanes 2, 3, 4, and 5). Negative controls included normal human sera (lane 1), sera from patients with cerebellar degeneration due to other causes (lane 7), patients with ovarian tumors (lane 8), and patients with ovarian tumors and cerebellar degeneration but no anti-Yo antibody (lanes 6 and 9) and serum from a patient with another antibody-associated paraneoplastic syndrome (anti-Ri) (lane 10).

ELISA for Anti-Yo Sera. The routine assay for the detection of anti-Yo antibodies involves immunohistochemical and Western blot analysis of human cerebellar tissue. Sera were defined as anti-Yo if they reacted specifically with Purkinje cells and identified a major species of 62 kDa on Western blot analysis. Western blot analysis was essential since we have encountered other sera that react with Purkinje cells but do not identify a 62-kDa protein (12). Screening for this antibody in a large patient population will require a simpler diagnostic assay that can be performed by clinical laboratories. Toward this end we have established an ELISA based on the recombinant CDR-62 antigen. Partially purified preparations of the pY2 fusion protein were immobilized on 96-well microtiter plates and incubated with the same group of sera (IgG, 2 μg/ml) utilized in Fig. 3*B*. All four anti-Yo sera were clearly positive whereas all negative sera were less than 0.02 OD_{405} unit (Fig. 4).

Sequence Analysis of pY1 and pY2. Sequence analysis of pY2 revealed a large open reading frame of 431 amino acids that was in-frame with the AUG initiation codon of β-galactosidase. The predicted molecular mass of this open reading frame (54 kDa) agrees well with that observed in Fig. 3*A*. Sequence analysis of pY1 confirmed that it overlapped with the 5′ end of pY2 and provided another 243 nucleotides of 5′ sequence. Together pY1 and pY2 yielded a composite open reading frame of 510 amino acids (Fig. 5). We have not yet established the N-terminal AUG codon. Secondary structure analysis revealed a highly hydrophilic protein with extensive regions of α-helix. The most conspicuous feature was a leucine-zipper motif found at residues 171–192. This motif, consisting of a heptad repeat of leucine residues forming an amphiphilic α-helix, is a distinctive feature of proteins that bind to DNA as a hetero- or homodimer (13, 14). The amphiphilic nature of the leucine zipper found in CDR-62 is illustrated in a helical wheel analysis (Fig. 6). A thin ridge of hydrophobicity is evident down the axis of the putative

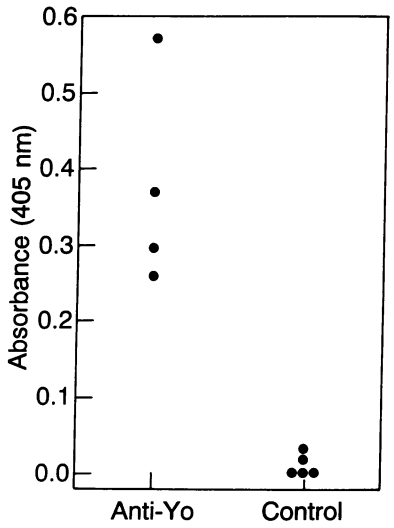

FIG. 4. ELISA using pY2 fusion protein. Partially purified pY2 fusion protein was immobilized to 96-well microtiter plates and serum reactivity was assayed. Each dot corresponds to a single determination.

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                  20                  40                  60
                   *                   *                   *
SAAGPNGAEAAQRSLGGGAS RPRAALAEGGGAGEEPGAAA EAGRRRGPLPLEDPAEMLAE

                  80                  100                 120
                   *                   *                   *
NLVEEFEMKEDEPWYDHQDL QQDLQLAAELGKTLLDRNTE LEDSVQQMYTTNQEQLQEIE

                                                >LEUZIP
                                                    |
                 140                 160            |    180
                   *                   *            |     *
YLTKQVELLRQMNEQHAKVY EQLDVTARELEETNQKLVAD SKASQQKILSLTETIECLQT

                 200                 220                 240
                   *                   *                   *
NIDHLQSQVEELKSSGQGRR SPGKCDQEKPAPSFACLKEL YDLRQHFVYDHVFAEKITSL

                 260                 280                 300
                   *                   *                   *
QGQPSPDEEENEHLKKTVTM LQAQLSLERQKRVTMEEEYG LVLKENSELEQQLGATGAYR

                 320                 340                 360
                   *                   *                   *
ARALELEAEVAEMRQMLQSE HPFVNGVEKLVPDSLYVPFK EPSQSLLEEMFLTVPESHRK

                 380                 400                 420
                   *                   *                   *
PLKRSSSETILSSLAGSDIV KGHEETCIRRAKAVKQRGIS LLHEVDTQYSALKVKYEELL

                 440                 460                 480
                   *                   *                   *
KKCQEEQDSLSHKAGRPPGC SQGPDWSDAQSEPVASGWEL ASVNPEPVSSPTTPPEYKAL

                 500
                   *
FKEIFSCIKKTKQEIDEQRT KYRSLSSHS
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FIG. 5. Composite open reading frame specified by pY1 and pY2 is shown in single-letter amino acid code. The leucine-zipper domain is shown and the participating leucine residues are highlighted by a dot.

α-helix. In addition by allowing the substitution of serine-164 and histidine-137, a "super" leucine zipper stretches from residues 122 to 170 in complete register with the leucine zipper at residues 171–192. Helical wheel analysis of this "super" leucine zipper also revealed a clear hydrophobic ridge down the axis of the α-helix. Preliminary experiments have shown that CDR-62 can in fact bind to DNA. By assaying CDR-62 by Western blot analysis, we have shown that the protein present in cytoplasmic extracts of HeLa cells binds strongly (elutes at 0.5 M NaCl) to native DNA-cellulose

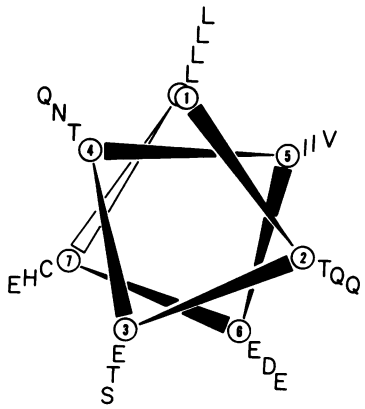

FIG. 6. Helical wheel analysis of the leucine-zipper domain. The analysis starts with Leu-171 and ends at Leu-192. The helical wheel consists of seven spokes corresponding to the fit of seven amino acids into every two α-helical turns. The single-letter amino acid code is used.

(H.F.-S. and H.M.F., unpublished data). CDR-62 does not appear to contain the characteristic basic DNA binding domain found in many leucine-zipper proteins. We note however the presence of two putative DNA binding motifs (a SPKK protein site at codon 201 and a zinc finger at codons 205–231) adjacent to the leucine zipper. Also consistent with the function of the protein as a transcription factor is the presence of a highly acidic (pI 3.4) activating domain between residues 52 and 80. Leucine-zipper proteins in general display little homology in the amino acids found between the leucine residues. In this case we note the significant amino acid sequence homology between a leucine-zipper element found in CDR-62 (LQTNIDHL) and that found in the leucine zipper present in c-Fos (LQTEIANL) (15).

DISCUSSION

We have screened a human expression library and isolated cDNA clones that encode an epitope recognized by the sera of patients with antibody-associated paraneoplastic cerebellar degeneration. On the basis of the epitope-selection analysis, we have concluded that these cDNAs correspond to the major antigen CDR-62. There is a possibility that the cDNAs encode an epitope shared with CDR-62 but do not correspond to the protein itself. Antibodies specifically raised against the fusion protein will definitively answer this question. Irrespective of the true identity of the cDNAs, they encode an antigenic species that is uniquely recognized by anti-Yo sera. In contrast to the previously cloned minor antigen (CDR-34), the fusion proteins encoded by the present cDNAs are highly reactive with anti-Yo sera (detectable binding is observed at an IgG concentration of 0.2 μg/ml). The fusion protein encoded by these clones provides the most sensitive assay for the detection of anti-Yo sera. Herein we demonstrate the feasibility of an ELISA-based simple diagnostic assay for this clinically important syndrome.

The most conspicuous structural feature of CDR-62 is a leucine-zipper motif. We believe that the presence of this feature and of putative DNA binding domains indicates that this protein may play a role in the regulation of gene expression. It will be crucial to isolate the DNA sequence recognized by CDR-62. We note the similarity of the leucine-zipper element between CDR-62 and c-Fos. It is conceivable that CDR-62 may interact with the same family of proteins that interact with c-Fos (15). There is no obvious similarity between CDR-62 and the previously cloned minor antigen CDR-34. They are clearly different gene products since CDR-34 resides on chromosome X (4) whereas CDR-62 resides on chromosome 16 (M. Siniscalco, H.M.F., and J.B.P., unpublished data). CDR-34 is an unusual protein consisting almost entirely of tandem repeats of a 6-amino acid consensus sequence L/FLEDVE. Such tandem repetition gives rise to a number of single (Leu-Leu) zipper elements. We speculate that anti-Yo sera specifically recognizes leucine-zipper elements. This speculation is further supported by the isolation (from an expression library) of another gene product (CDIII) recognized by anti-Yo sera that contains a leucine-zipper element (H.F.-S. and H.M.F., unpublished data).

Our current hypothesis concerning the pathological mechanisms of the syndrome is that cerebellar dysfunction arises from an immune response directed against the cerebellum but provoked by the aberrant expression of the neural antigen in tumor tissue. There is no direct proof of this model. Injection of anti-Yo antibodies into experimental animals has failed to reproduce the syndrome. The availability of the recombinant CDR-62 antigen may permit the generation of an appropriate immune response in experimental animals and hopefully create an animal model. In addition, specific expression of CDR-62 in patient's tumor samples can now be examined. The other fascinating aspect of this syndrome is that these patients exhibit an exaggerated immune response to their tumor tissue. Tumor tissue (presumably the CDR-62 antigen) is clearly perceived as foreign by the patient's immune system. There are at least two models that may help us to understand this phenomenon. Firstly, it may be that the CDR-62 protein is normally specifically restricted in its expression to brain tissue. The brain is an immunologically privileged site (16, 17). The implication is that the *de novo* expression of a brain protein in extraneural tumor tissue may provoke an intense immune response. In the second model we suggest that the CDR-62 antigen is expressed normally in extraneural tissue but it undergoes a somatic mutation. In view of the highly hydrophilic α-helical structure of CDR-62, it is reasonable to suppose that even a single-amino acid change may drastically affect the structure of the protein. The abnormal epitopes thus created may result in CDR-62 being perceived as foreign by the immune system. A prerequisite for this model is that all the tumors express the mutated derivative of CDR-62. The simplest way in which this may occur is if the putative mutation of CDR-62 converts the protein into an oncogene. The transforming version of CDR-62 may itself contribute to the development of the tumor.

Note Added in Proof. We note the similarity between the C terminus of the sequence reported herein and that of Sakai *et al.* (18).

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1. Henson, R. & Urich, H. (1982) *Cancer and the Nervous System* (Blackwell, Oxford).
2. Brain, L. & Wilkinson, M. (1965) *Brain* **88,** 465–478.
3. Furneaux, H. & Posner, J. (1990) *Res. Publ. Assoc. Res. Nerv. Ment. Dis.* **68,** 187–219.
4. Anderson, N. E., Rosenblum, M. K., Graus, F., Wiley, R. G. & Posner, J. B. (1988) *Neurology* **38,** 1391–1398.
5. Furneaux, H., Rosenblum, M., Dalmau, J., Wong, E., Woodruff, P., Graus, F. & Posner, J. (1990) *N. Engl. J. Med.* **322,** 1844–1851.
6. Furneaux, H., Reich, L. & Posner, J. (1990) *Neurology* **40,** 1085–1091.
7. Cunningham, J., Graus, F., Anderson, N. & Posner, J. (1986) *Neurology* **36,** 1163–1168.
8. Dropcho, E. J., Chen, Y.-T., Posner, J. B. & Old, L. J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 4552–4556.
9. Furneaux, H. M., Dropcho, E. J., Barbut, D., Chen, Y.-T., Rosenblum, M. K., Old, L. J. & Posner, J. B. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2873–2877.
10. Maniatis, T., Fritsch, B. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
11. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4352.
12. Darnell, R., Furneaux, H. & Posner, J. (1989) *Neurology* **39,** Suppl. 1, 385.
13. Landschulz, W. H., Johnson, P. F. & McKnight, S. L. (1988) *Science* **240,** 1759–1764.
14. Vinson, C., Sigler, P. & McKnight, S. (1989) *Science* **246,** 911–916.
15. Gentz, R., Rauscher, F. I., Abate, C. & Curran, T. (1989) *Science* **243,** 1695–1699.
16. Lampson, L. (1987) *Trends Neurosci.* **10,** 211–216.
17. Medawar, P. (1948) *Br. J. Exp. Pathol.* **29,** 58–69.
18. Sakai, K., Mitchell, D. J., Tsukamoto, T. & Steinman, L. (1990) *Ann. Neurol.* **28,** 692–698.