ponents of Bottenstein *et al.* (*10*) supported spinal cord neuronal survival during the first week in culture, as also observed by Skaper *et al.* (*11*), but not beyond 2 weeks. Addition of N1 components to the active serum fraction did not alter the relation between fraction concentration and neuronal survival measured at 21 days.

In conclusion, acid gel-filtration of serum from a variety of mammals yields a fraction that reliably supports the long-term survival of rat central neurons and several other cell types in vitro, without concomitantly stimulating cellular proliferation. These properties are of obvious practical importance to studies requiring neuron-rich cultures, especially since the metabolic inhibitors traditionally used to limit nonneuronal cell proliferation can alter neuronal properties (*12*). Biochemical and biological characteristics of the active serum factor do not appear to correspond to those of any well-defined growth factor (*13*). Since the concentration of this factor in the sera we tested was more than sufficient to support neuronal survival in vitro, it is likely that the factor contributes to neuronal survival in vivo.

LAWRENCE M. KAUFMAN
JOHN N. BARRETT

Department of Physiology and Biophysics, University of Miami, Miami, Florida 33101

References and Notes

1. For review, see S. Vinores and G. Guroff, *Annu. Rev. Biophys. Bioeng.* **9**, 223 (1980).
2. G. D. Fischbach and P. G. Nelson, in *Handbook of Physiology, section 1, The Nervous System*, vol. 1, *Cellular Biology of Neurons*, E. R. Kandel, Ed. (Williams & Wilkins, Baltimore, 1977), p. 719.
3. Costar 24-well cluster dishes were coated with a thin layer of rat tail collagen dissolved in 1 m*M* acetic acid, air dried, washed for 30 minutes with a solution of poly-L-lysine (10 μg/ml, Sigma type 1-B) buffered to *p*H 9.0, and finally rinsed with water [E. Yavin and Z. Yavin, *J. Cell Biol.* **62**, 540 (1974)].
4. Culture medium modified from DMEM by: (i) the deletion of glycine, glutamine, pyruvate, and ferric nitrate; (ii) reduction of phenylalanine to 2 mg/liter, tryptophan to 2 mg/liter, and glucose to 40 mg/liter; (iii) addition of alanine (150 mg/liter), proline (40 mg/liter), cyanocobalamin (1 mg/liter), carnitine (1 mg/liter), *p*-aminobenzoic acid (0.5 mg/liter), succinate (10 mg/liter), citrate (20 mg/liter), α-β-glycerophosphate (100 mg/liter), galactose (200 mg/liter), fructose (100 mg/liter), and mannose (50 mg/liter). Motivations for this change of nutrient medium included elimination of potential neurotransmitters and provision of a more complete supply of small nutrient molecules normally found in CSF. Use of this CSF-like medium avoided a late neuronal die-off that frequently occurred after 15 days in vitro when the cells were maintained throughout the experiment in the active serum fractions (see text) equilibrated in DMEM.
5. B. R. Ransom, E. Neale, M. Henkart, P. N. Bullock, P. G. Nelson, *J. Neurophysiol.* **40**, 1132 (1977).
6. To check our molecular weight estimate for maximum neuronal survival promoting activity, we rechromatographed the active fraction from the acid column described above on a high-performance liquid chromatography column (LKB, TSK G 3000 SW) at *p*H 7.0. Again, peak activity eluted at an apparent molecular weight of 55,000.
7. The lack of proliferation of nonneuronal cells in medium containing the active serum fraction (horizontal bar, Fig. 2A) is not due to toxic effects, since adding whole serum to medium containing this fraction results in rapid proliferation of glia and fibroblasts.
8. B. H. Ginsberg, C. R. Kahn, J. Roth, K. Megyesi, G. Bauman, *J. Clin. Endocrinol. Metab.* **48**, 43 (1979).
9. L. B. Dribin and J. N. Barrett, *Dev. Biol.* **74**, 184 (1980); R. G. Smith and S. H. Appel, *Soc. Neurosci. Abstr.* **7**, 144 (1981); R. W. Oppenheim, J. L. Maderdrut, D. J. Wells, *J. Comp. Neurol.* **210**, 174 (1982).
10. J. E. Bottenstein, S. D. Skaper, S. S. Varon, G. H. Sato, *Exp. Cell Res.* **125**, 183 (1980).
11. S. D. Skaper, R. Adler, S. Varon, *Dev. Neurosci.* **2**, 233 (1979).
12. V. Argiro and M. Johnson, *Soc. Neurosci. Abstr.* **7**, 346 (1981).
13. R. A. Bradshaw and J. S. Rubin, *J. Supramol. Struct.* **14**, 183 (1980).
14. M. Bradford, *Anal. Biochem.* **72**, 248 (1976). Reagents from Bio-Rad, BSA as standard.
15. We thank E. Barrett for help in preparing the manuscript and D. Nonner and S. Barca for technical help. Supported by NIH grants NS12207 and NS07044 and the National Parkinson Foundation.

4 March 1983

Simple Repeat Array in Epstein-Barr Virus DNA Encodes Part of the Epstein-Barr Nuclear Antigen

Abstract. *The size of the Epstein-Barr virus (EBV) nuclear antigen (EBNA) in cells infected with different EBV isolates varies directly with the size of the EBV triplet repeat array, IR3. The isolate with the largest IR3 fragment has approximately 170 more codons than the isolates with the smallest IR3 fragment; it encodes an EBNA which is approximately 17,000 daltons larger than the smallest EBNA. The EBV IR3 encodes part of a 2-kilobase exon of a latently infected cell messenger RNA which must be translated into a repetitive amino acid domain of EBNA.*

Epstein-Barr virus (EBV) infection induces proliferation of human lymphocytes (*1*). The infected lymphocyte is frequently nonpermissive for the expression of early or late viral replicative functions. An Epstein-Barr intranuclear antigen (EBNA), presumed to be specified by EBV, can be detected in growth-transformed cells with human immune serum (that is, from infected patients) by means of anticomplementary immunofluorescence (ACIF). The antigen is also present in cells from Burkitt's lymphoma (*2, 3*) and nasopharyngeal carcinoma (*4*), two human malignant diseases in which EBV may be an etiologic agent. The entire virus genome persists in the growth-transformed cell as circular episomes (*5, 6*) and as integrated viral DNA (*7*); it consists of single-copy (unique, U), direct terminal repeat (TR) elements, and direct internal repeat (IR) elements in the order TR-U1-IR1-U2-IR2-U3-IR3-U4-IR4-U5-TR (from left to right) (*8*). Messenger RNA's [3.0 and 3.7 kilobases (kb)] are transcribed from left to right from the IR1–U2 and U3–IR3–U4 regions of EBV DNA; and a 2.7-kb RNA is transcribed from right to left from U5 (*8–12*). The sequence IR3 consists of direct repeats of GGG, GCA, GGA (G, guanine; A, adenine; C, cytosine) in two nonanucleotides or one hexanucleotide repeat unit and encodes part of a 2-kb exon of the 3.7-kb latently infected cell RNA (*11*). The unusual triplet symmetry of this repeat through 708 base pairs (bp) and the transcription of this sequence in latently infected cells suggested that this repeat might encode part of a protein in a latently infected cell (*11*).

The nuclear antigen, EBNA, is the most conspicuous protein from a latently infected cell as judged by immunoblots (*13*) with ACIF-positive EBV immune human serum (*14–16*). These serums identify a 65,000-dalton (65K) protein in Raji cells and a 72K protein in B95-8 cells (*15, 16*). Since the EBV Bam HI K fragment that contains the IR3 simple triplet nucleotide repeat array is 0.3 kb shorter in Raji EBV DNA than in B95-8 EBV DNA, the EBNA protein could vary in size among samples of EBV as a consequence of variation in the length of the repeat (*11, 17*). We have been able to map with reasonable precision the IR3 exon and directly test the relation between IR3 and EBNA by determining the sizes of the IR3 repeat region and the EBNA protein in B95-8, in IB4 (which is a latently infected B95-8 transformant) (*18*), in Raji, and in two other cell lines carrying viruses whose IR3 regions differ substantially from those of B95-8 and Raji (*11*).

The relevant restriction endonuclease sites in EBV DNA are indicated in Fig. 1. In previous mapping of the 2-kb exon of the 3.7-kb RNA, separated strands of the Sal I F fragment of EBV DNA were utilized (*11*). In our experiments, the Bam HI K fragment was cloned into pKH47 to map the exon. The EBV DNA strand R (5′ end to the right in the map shown in Fig. 1) was chromatographed on oligodeoxyadenylate cellulose, and it was then hybridized to RNA from the IB4 cell line. The size of the Bam HI K DNA segment protected by IB4 cytoplasmic polyadenylated RNA is 2 kb and is identical in size to the Sal I F segment

protected by S1 nuclease. The orientation of the Bam HI K fragment within one recombinant pKH47 clone is such the leftward 2.8 kb attached to polydeoxythymidine. Hybridization of IB4 cytoplasmic polyadenylated RNA to this DNA also results in protection of a 2.0-kb segment. These results indicate that the 2.0-kb exon maps within the 2.8-kb Bam HI–Hind III fragment of Bam HI K. On the basis of the intensities of IR3 probe hybridizations to blots of the 3.7-kb RNA and to blots of known amounts of Bam HI K DNA, we estimate that there are three copies of the 3.7-kb RNA per IB4 cell.

Examination of Bam HI K fragments of EBV isolates showed that the sizes of the B95-8 and IB4 Bam HI K fragments were 5.0 kb, and the sizes of Raji, Lamont, and Cherry were 4.7, 4.8, and 5.2 kb, respectively (*11*). To demonstrate that this variability was due to differences in IR3 length, we treated these DNA's (i) with Hinf I, which cuts within Bam HI K 270 bp to the left and 170 bp to the right of IR3, or (ii) with Msp I, which cuts within Bam HI K 57 bp to the left and 11 bp to the right of IR3 (*11*) (Fig. 1). The sizes of the IR3 Hinf I fragments of Cherry, IB4, Lamont, and Raji, are, respectively, 1400, 1150, 990, and 910 bp (data not shown). The sizes of the respective IR3 Msp I fragments are Cherry, 1050 bp; IB4, 776 bp; Lamont, 630 bp; and Raji, 550 bp (Fig. 2A). Thus, all of the variation in size of the Bam HI K is due to variation within the Msp I fragment. The IR3 repeat is 708 bp of the 776-bp Msp I sequence B95-8 (*11*). The Raji and Lamont Msp I fragments are

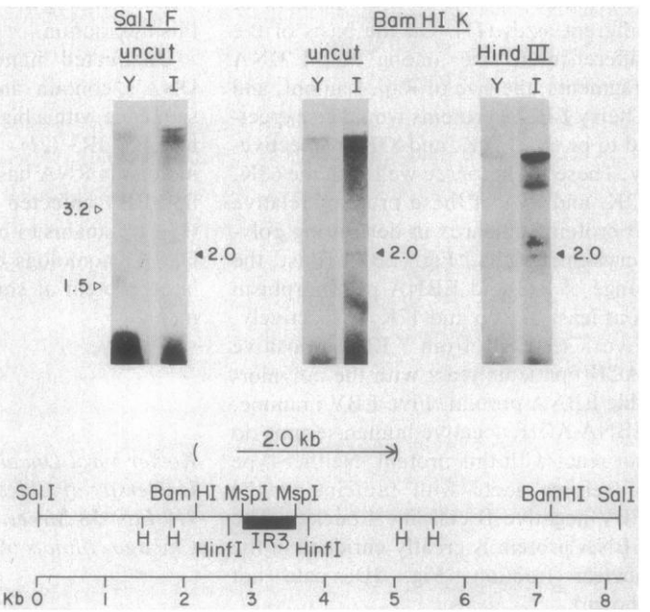

Fig. 1. The IR3 region (Sal I F fragment) of EBV DNA (*11*). Cytoplasmic polyadenylated RNA from IB4 was analyzed by hybridization to strand separated EBV Bam HI K or Sal I F DNA and S1 nuclease digestion (*9–12*). Restriction enzymes used to cut the EBV DNA sequences before strand separation and hybridization to RNA are indicated above the lanes and in the map at the bottom. The DNA between the two Bam HI sites shown is the Bam HI K fragment. After Hind III digestion of the plasmid containing the Bam HI K fragment, only EBV DNA sequences to the left of the first Hind III site in Bam HI K remained covalently attached to the binding site for oligodeoxyadenylate cellulose. *Y* indicates hybridizations with yeast RNA as controls, and *I* indicates hybridizations with IB4 cytoplasmic polyadenylated RNA. After S1 digestion DNA was subjected to electrophoresis on an alkaline gel, blotted onto nitrocellulose, and detected by hybridization to the ^{32}P-labeled IR3 Msp I fragment. Marker sizes were 3.2 and 1.5 kb. DNA's protected by IB4 RNA and not yeast RNA appeared at 2.0 kb. The band at 2.5 kb with Hind III cut Bam HI K was not seen in control lanes and may result from unspliced nuclear RNA extending from left of the Bam HI site to the 3′ end of the RNA. The RNA used for the experiments shown was contaminated with nuclear RNA (*9–12*). *H* indicates Hind III sites. The direction of transcription of the RNA is indicated by an arrow.

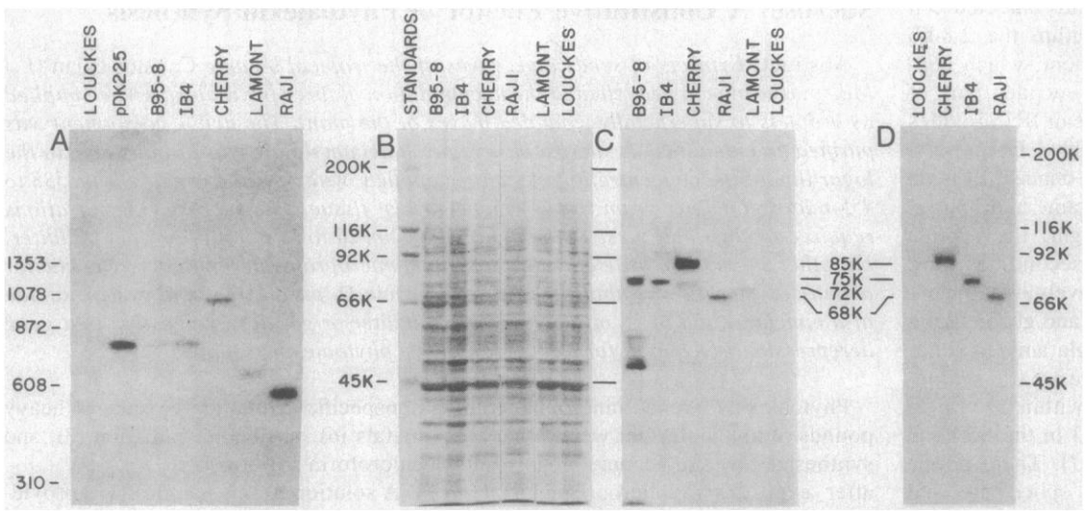

Fig. 2. Size of IR3 fragment and EBNA in EBV-infected cell lines. (A) IR3 polymorphism. To determine the size of the IR3 sequence in other EBV isolates, the 776 bp B95-8 Msp I IR3 fragment was labeled by nick translation and hybridized to a Southern blot of Msp I digested cell DNA's. Total infected cell DNA's (2.5 μg) were digested with Msp I, subjected to electrophoresis on a 1.4 percent agarose gel and transferred to nitrocellulose. Lane 1, Loucke; lane 2, 776 bp of IR3 Msp I fragment from pDK225 mixed with human cell DNA; lane 3, B95-8; lane 4, IB4; lane 5, Cherry; lane 6, Lamont; lane 7, Raji. Some DNA's were diluted with human cell DNA to normalize for EBV DNA copy number. Size markers in base pairs are Hae III fragments of the ϕX174 replicative form DNA. (B to D) EBNA protein polymorphism. Immunoblots of total cellular proteins isolated from EBV productively infected (B95-8), latently infected (IB4, Cherry, Raji and Lamont), or EBV-negative (Loucke) B lymphocyte cell lines (*9–11*). Samples were prepared by washing 3×10^7 cells with phosphate-buffered saline, centrifuging, and solublizing the pellet in 200 μl of buffer consisting of 6 percent sodium dodecyl sulfate (SDS), 2 m*M* β-mercaptoethanol, 140 m*M* tris-HCl (*p*H 7.0), 2 percent glycerol, and 0.07 percent bromophenol blue. The samples were heated at 100°C for 5 minutes, subjected to electrophoresis on an 8.5 percent SDS-polyacrylamide slab gel (*19*), and transferred to a nitrocellulose sheet (*13*). The nitrocellulose sheet was then divided and either stained with Coomassie brilliant blue (B) or immunostained (*13*) with a 1:60 dilution of EBNA-positive human serum No. 334 (ACIF titer > 1:1280) and ^{125}I-labeled protein A (Amersham) (C). Nuclear proteins were prepared by Nonidet P40 lysis (*10*) and immunostained with an EBNA-positive serum No. 457 (ACIF-titer > 1:1280). Molecular size standards are indicated, as well as the size of EBNA, for each cell line.

140 and 220 bp smaller, respectively, than B95-8 and must therefore contain a smaller IR3. Since the B95-8 Msp I fragment is larger because of a longer IR3, the Msp I fragment of Cherry is likely to be largest in that it contains the longest IR3.

The size of the EBNA protein in cell lines infected with each of the EBV isolates was determined by incubating EBNA positive human serums with immunoblots of cell protein extracts (Fig. 2, B to D). The size of B95-8 and IB4 EBNA are 75K; near the size previously determined for B95-8 with markers of different sizes (*15*). On the basis of the difference in size among Msp I DNA fragments, the size of Raji, Lamont, and Cherry EBNA proteins would be expected to be 69K, 71K, and 83K, respectively. These values agree well with the 68K, 72K, and 85K of these proteins relative to protein standards in denaturing polyacrylamide gels (Fig. 2B). Thus, the range of IR3 and EBNA polymorphism is at least 500 bp and 17K, respectively. Five serums from EBNA-positive (ACIF) patients react with the polymorphic EBNA protein. Five EBV immune, EBNA ACIF negative human serums do not react with this protein. Neither type of serum reacts with proteins of the EBV-negative B cell line Louckes. The EBNA protein is greatly enriched in the nuclear fraction (Fig. 2D; data not shown).

These data indicate that (i) the 2-kb exon of the 3.7-kb latently infected cell RNA maps entirely within the 2.8-kb Bam HI–Hind III fragment, which contains IR3 and (ii) IR3 encodes part of EBNA. Variation in size of IR3 provides the mechanism by which EBNA polymorphism is generated. Since IR3 is an entirely open reading frame and is transcribed from left to right, the peptide encoded by IR3 first, second, or third reading frames, respectively, would consist of serine, arginine, and glycine; glycine and alanine; or glutamine, glutamate, and glycine. There are translational termination codons within 250 bp to the left and right of IR3 in the first and third reading frames (*11*). There is one possible, but unlikely, splice site that could eliminate stop codons (*11, 19*). If this unlikely candidate splice acceptor site is not functional, the first and third reading frames are excluded since the size of EBNA is approximately 80K and EBNA is encoded in part by IR3. The second reading frame encodes a glycine alanine copolymer which would then represent approximately 19K of the 75K B95-8 EBNA protein. Further evidence that this region encodes EBNA is that transfection of the Bam HI K fragment or of its leftward Bam HI–Hind III subfragment, which contains IR3 (Fig. 1), into L cells has been reported to induce an intranuclear antigen detectable only with EBNA-positive human serums (*20*).

Our result indicates that EBNA has a core of simple amino acid composition. Because of its binding to chromosomes, EBNA may participate in the regulation of cell growth (*15, 16*). The identification of a copolymer domain in EBNA is a step in defining the protein component of this interaction.

Uninfected human and mouse cell DNA's contain an interspersed repeat sequence with a high degree of homology to EBV IR3 (*21*). Although no IR3 homologous RNA has been detected in the non-EBV infected Louckes B cell line (*11*), it remains to be seen whether cellular IR3 homologs encode an EBNA-related protein at some stage of development.

KEVIN HENNESSY
MARK HELLER
VICKY VAN SANTEN
ELLIOTT KIEFF

Kovler Viral Oncology Laboratories,
University of Chicago,
910 East 58 Street,
Chicago, Illinois 60637

References and Notes

1. J. H. Pope, M. K. Horne, W. Scott, *Int. J. Cancer* **3**, 857 (1968).
2. B. M. Reedman and G. Klein, *ibid.* **11**, 499 (1973).
3. ———, J. H. Pope, M. K. Walters, J. Hilgers, S. Singh, B. Johansson, *ibid.* **13**, 755 (1974).
4. G. Klein, B. C. Giovanella, T. Lindahl, P. J. Fialkow, S. Singh, J. Stehlin, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4737 (1974).
5. M. Nonoyama and J. S. Pagano, *Nature (London) New Biol.* **233**, 103 (1971).
6. T. Lindahl, A. Adams, G. Bjursell, G. W. Bornkamm, C. Kaschka-Dierich, U. Jehn, *J. Mol. Biol.* **102**, 511 (1976).
7. A. Henderson, S. Ripley, M. Heller, E. Kieff, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1987 (1983).
8. E. Kieff *et al.*, *J. Infect. Dis.* **146**, 506 (1982).
9. V. van Santen, A. Cheung, E. Kieff. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1930 (1981).
10. V. van Santen, A. Cheung, M. Hummel, E. Kieff, *J. Virol.* **46**, 424 (1983).
11. M. Heller, V. van Santen, E. Kieff, *ibid.* **44**, 311 (1982).
12. V. van Santen, thesis, University of Chicago (1982).
13. W. Burnette, *Anal. Biochem.* **112**, 195 (1980).
14. S. Slovin *et al.*, *J. Immunol.* **127**, 585 (1981).
15. B. Strnad, T. Schuster, R. Hopkins, R. Neubauer, H. Rabin, *J. Virol.* **38**, 996 (1981).
16. T. C. Spelsberg, T. B. Sculley, G. M. Pikler, J. A. Gilbert, G. R. Pearson, *ibid.* **43**, 555 (1982).
17. In fact, simple repeat arrays such as IR3 frequently vary in length; G. P. Smith, *Science* **191**, 528 (1976).
18. W. King, A. L. T. Powell, N. Raab-Traub, M. Hawke, E. Kieff, *J. Virol.* **36**, 506 (1980).
19. S. Mount, *Nucleic Acids Res.* **10**, 459 (1982).
20. W. Summers, E. Grogan, D. Shedd, M. Robert, C. Lui, G. Miller, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5688 (1982).
21. M. Heller, A. Henderson, E. Kieff, *ibid.*, p. 5916.
22. Supported by PHS grants CA 19264 and CA 17281, American Cancer Society grant ACS MV32H, NIH National Research Service Awards GM 07183 (to K.H.) and AI 07099 (to M.H.), and by a Faculty Research Award from the American Cancer Society (to E.K.). We thank Professor W. Henle and G. Lenoir for serums.

9 March 1983; revised 11 April 1983

Sucrose: A Constitutive Elicitor of Phytoalexin Synthesis

Abstract. *Extracts of seeds and leaves of the tropical legume* Cajanus cajan *(L.) Millsp. (the pigeon pea) elicited the accumulation of three phytoalexins when applied as droplets to superficially wounded leaves of the plant. The active component was purified and identified as sucrose. Phytoalexin accumulation was proportional to the logarithm of the concentration of sucrose applied, with maxima ranging from 338 to 455 micrograms per gram (fresh weight) of leaf tissue. The sucrose concentrations required to elicit half these amounts ranged from 20 to 35 micrograms per milliliter, but other sugars had little effect even at 1000 micrograms per milliliter. The elicitor activity of sucrose was abolished by actinomycin D, puromycin, and cycloheximide at a concentration of 10 micrograms per milliliter or greater, suggesting that gene derepression is required for expression of the phytoalexin response.*

Phytoalexins are antimicrobial compounds of low molecular weight that are synthesized by and accumulate in plants after exposure to microorganisms (*1*). The role of these compounds as antiparasitic agents in some plants is supported by considerable evidence (*2, 3*), but little is known about the mechanisms that control their production. They may be synthesized specifically in response to avirulent races of pathogenic fungi (*4*) but not to virulent races of the same organism (*4, 5*), and yet they also accumulate as a result of treatment with such nonspecific agents as the salts of heavy metals (*6*), ultraviolet radiation (*7*), and chloroform vapor (*8*).

A solution to this paradox was provided by Bailey (*9*), who showed that plants contain a constitutive compound that may function as a phytoalexin elicitor (*10*). It is thought that this compound diffuses from damaged cells and elicits phytoalexin synthesis in adjacent healthy cells. Thus damage, whether caused by avirulent races of pathogens or by less subtle means, may trigger phytoalexin synthesis. Recent work (*11*–