70. J. van der Veen and L. Bots, *Arch. Gesamte Virusforsch.* **81**, 230 (1958).
71. W. A. Nelson-Rees, *Prog. Clin. Biol. Res.* **26**, 25 (1978).
72. R. S. Verma, H. Dosik, K. A. Lavappa, *J. Hered.* **68**, 287 (1977).
73. G. Seman, S. J. Hunter, R. C. Miller, L. Dmochowski, *Cancer* **37**, 1814 (1976).
74. G. Pinaev, D. Bandyopadhyay, O. Glebov, V. Shangbag, G. Johansson, P. O. Albertsson, *Exp. Cell Res.* **124**, 191 (1979).
75. M. A. McKinlay, D. E. Wilson, B. Harrison, S. Povey, *J. Natl. Cancer Inst.* **64**, 241 (1980).
76. P. M. Kraemer, L. L. Deaven, H. A. Cressman, J. A. Steinkamp, D. F. Peterson, *Cold Spring Harbor Symp. Quant. Biol.* **38**, 133 (1973).
77. E. E. Miller, *In Vitro* **12**, 331 (1976).
78. W. A. Nelson-Rees and R. R. Flandermeyer, *Science* **195**, 1343 (1977).
79. W. K. Heneen, *Hereditas* **82**, 217 (1976).
80. C. F. Hogman, *Exp. Cell Res.* **21**, 137 (1960).
81. A. W. Moyer, R. Wallace, H. R. Cox, *J. Natl. Cancer Inst.* **33**, 227 (1964).
82. P. D. Noguchi, J. B. Johnson, R. O'Donnell, J. C. Petricciani, *Science* **199**, 980 (1978).
83. G. R. Mikhailova *et al.*, *Cytologia* **19**, 786 (1977).
84. J. Y. Chou, B. D. Weintraub, S. W. Rosen, J. Whangpeng, H. D. Sussman, J. R. Houghom, J. C. Robinson, *In Vitro* **12**, 589 (1976).
85. E. L. Springer, A. J. Hackett, W. A. Nelson-Rees, *Int. J. Cancer* **17**, 407 (1976).
86. W. A. Nelson-Rees, L. Hunter, G. J. Darlington, S. J. O'Brien, *Cytogenet. Cell Genet.* **27**, 216 (1980).
87. M. Lippman, G. Bolan, K. Huff, *Cancer Res.* **36**, 4595 (1976); *ibid.*, p. 4602; *ibid.*, p. 4610.
88. R. Cailleau, *Cancer Res.* **20**, 837 (1960).
89. S. H. Hsu, B. E. Schacter, N. L. Delaney, T. B. Miller, V. A. McKusick, R. H. Kennett, J. G. Bodmer, D. Young, W. F. Bodmer, *Science* **191**, 392 (1976).
90. S. Povey, D. A. Hopkinson, H. Harris, L. M. Franks, *Nature (London)* **264**, 60 (1976).
91. M. J. Siciliano, P. E. Barker, R. Cailleau, *Cancer Res.* **39**, 919 (1979).
92. S. Pathak, M. J. Siciliano, R. Cailleau, C. L. Wiseman, T. C. Hsu, *J. Natl. Cancer Inst.* **62**, 263 (1979).
93. S. Kit, D. R. Dubbs, P. M. Frearson, *Int. J. Cancer* **1**, 19 (1966).
94. K. E. Hampel and A. Levan, *Hereditas* **51**, 315 (1964).
95. This publication.
96. J. J. Maio and L. L. De Carli, *Nature (London)* **196**, 600 (1962).
97. R. Kennett, B. Hampshire, B. Bengtsson, W. F. Bodmer, *Tissue Antigens* **6**, 80 (1975).
98. M. A. Pellegrino, S. Ferrone, A. Pellegrino, *Proc. Soc. Exp. Biol. Med.* **139**, 484 (1972).
99. R. Glaser, G. Lenoir, S. Ferrone, M. A. Pellegrino, G. de-Thé, *Cancer Res.* **37**, 2291 (1977).
100. W. A. Nelson-Rees, R. R. Flandermeyer, D. W. Daniels, *Science* **209**, 719 (1980).
101. J. Fogh and G. Trempe, in *Human Tumor Cells in Vitro*, J. Fogh, Ed. (Plenum, New York, 1975), pp. 115–159.
102. J. Fogh, W. C. Wright, J. D. Loveless, *J. Natl. Cancer Inst.* **58**, 209 (1977).
103. J. Fogh, J. M. Fogh, T. Orfeo, *ibid.* **59**, 221 (1977); corrected in *ibid.*, p. 1035.
104. W. A. Nelson-Rees, *ibid.* **63**, 537 (1979).
105. A. Leibovitz, W. C. Wright, S. Pathak, M. J. Siciliano, W. P. Daniels, H. Fogh, J. Fogh, *ibid.*, p. 635.
106. P. R. Herrick, G. W. Bauman, D. J. Merchant, M. C. Shearer, J. C. Shipman, R. G. Brackett, *In Vitro* **6**, 143 (1970).
107. J. Fogh, N. B. Holmgren, P. P. Ludovici, *ibid.* **7**, 26 (1971).
108. K. A. Rafferty, Jr., *Adv. Cancer Res.* **21**, 249 (1975).
109. R. Hay, Ed., *Catalogue of Strains II* (American Type Culture Collection, Rockville, Md., ed. 2, 1979).
110. R. W. Veltri, L. W. Heyl, P. M. Sprinkle, *Proc. Soc. Exp. Biol. Med.* **125**, 1246 (1967).
111. Y. Hinuma, M. Konn, J. Yamaguchi, D. J. Wudarski, J. R. Blakeslee, Jr., J. T. Grace, Jr., *J. Virol.* **1**, 1045 (1967).
112. B. R. Conner, S. Ferrone, M. A. Pellegrino, R. Glaser, *In Vitro* **16**, 446 (1980).
113. M. A. Epstein, B. G. Achong, Y. M. Barr, B. Zajac, G. Henle, W. Henle, *J. Natl. Cancer Inst.* **37**, 547 (1966).
114. M. A. Epstein and Y. M. Barr, *Lancet* **1964-I**, 252 (1964).
115. A. A. Creasey, H. S. Smith, A. J. Hackett, K. Fukuyama, W. L. Epstein, S. H. Madin, *In Vitro* **15**, 342 (1979).
116. A. E. Greene, J. Charney, W. W. Nichols, L. L. Coriell, *ibid.* **7**, 313 (1972).
117. T. D. C. Grace, *Nature (London)* **195**, 788 (1962).
118. L. S. Kucera and J. Simonson, *J. Natl. Cancer Inst.* **53**, 415 (1974).
119. C. C. Huang and H. F. Clark, *Can. J. Genet. Cytol.* **9**, 449 (1967).
120. M. Gravell and R. G. Malsberger, *Ann. N.Y. Acad. Sci.* **126**, 555 (1965).
121. J. J. Freed, L. H. Toji, A. E. Greene, L. S. Kucera, J. Simonson, S. J. O'Brien, H. Coon, *J. Natl. Cancer Inst.* **60**, 493 (1978).
122. J. Lightbody, S. E. Pfeiffer, P. L. Kornblith, H. Hershman, *J. Neurobiol.* **1**, 411 (1970).
123. P. Benda, J. Lightbody, G. Sato, L. Levine, W. Sweet, *Science* **161**, 370 (1968).
124. A. C. Stoolmiller, A. Dorfman, G. H. Sato, *ibid.* **178**, 1308 (1972).
125. I. Benjamin and H. Pinkerton, *Cancer Lett.* **1**, 203 (1976).
126. F. Taguchi, M. Toba, A. Tada, *Arch. Virol.* **60**, 347 (1979).
127. A. D. Stock and T. C. Hsu, *Chromosoma* **43**, 211 (1973).
128. W. A. Nelson-Rees, D. W. Daniels, R. R. Flandermeyer, *Arch. Virol.* **67**, 101 (1981).
129. J. C. Long, P. C. Zamecnik, A. C. Aisenberg, L. Atkins, *J. Exp. Med.* **145**, 1484 (1977).
130. L. V. Melendez *et al.*, *Lab. Animal Care* **19**, 372 (1969).
131. N. L. Harris, D. L. Gang, S. C. Quay, S. Poppema, P. C. Zamecnik, W. A. Nelson-Rees, S. J. O'Brien, *Nature (London)* **289**, 228 (1981).
132. E. Y. Lasfargues and L. Ozello, *J. Natl. Cancer Inst.* **21**, 1131 (1958).
133. P. D. Neuwald, C. Anderson, W. O. Salivar, P. H. Aldenderfer, W. C. Dermody, B. D. Weintraub, S. W. Rosen, W. A. Nelson-Rees, R. W. Ruddon, *ibid.* **64**, 447 (1980).
134. C. S. Stulberg in *Contamination in Tissue Culture*, J. Fogh, Ed. (Academic Press, New York, 1973), pp. 1–27.
135. M. V. Fernandes, *Z. Zellforsch.* **50**, 433 (1959).
136. V. Defendi, R. E. Billingham, W. K. Silvers, P. Moorhead, *J. Natl. Cancer Inst.* **25**, 359 (1965).
137. J. E. Shannon, personal communication (1981).
138. R. E. Stevenson, personal communication (1981).
139. N. J. Schmidt, personal communication (1981).
140. Much of the information in this report, some of it in greater detail, is available in *American Type Culture Collection, Catalogue of Strains II* (ATCC, Rockville, Md., ed. 3, 1981). We thank W. D. Peterson, Jr., for the G6PD mobility determinations and species-specific immunofluorescence tests on the cell cultures examined by us; S. J. O'Brien for work to determine allozyme genetic signatures on many of the cell cultures tested by us; and S. Ferrone for the HLA-A, B phenotyping of a number of cultures in our work. This work was performed under contract number YO1 CP8-0500 between the Office of Naval Research and the National Cancer Institute.

12 January 1981

Epstein-Barr Viral DNA: Infectivity for Human Placental Cells

Abstract. *Purified DNA of Epstein-Barr virus (EBV) is regularly infectious by means of the "calcium" method of transfection. Cultured human placental cells exposed to EBV DNA of two transforming strains, FF41 and B95, produce virus that is capable of converting normal B lymphocytes into established cell lines. After treatment with EBV (FF41) DNA and EBV (HR-1) DNA the placental cells display antigens associated with the productive viral cycle. The placental cells have not developed foci or other signs of morphologic transformation.*

Epstein-Barr virus (EBV), a human lymphotropic herpesvirus, is the cause of infectious mononucleosis and has been implicated in the etiology of three human cancers, nasopharyngeal carcinoma, Burkitt lymphoma, and immunoblastic lymphoma. The biologic behavior of the virus has been studied mainly in lymphocytes. Nearly all strains of EBV are capable of converting normal B lymphocytes of man and certain species of nonhuman primates into continuous (immortalized) cell lines (*1*). One exceptional nontransforming strain of the virus, called P3HR-1, undergoes an abortive cycle of replication in certain established lymphoblastoid cell lines of B cell origin (*2*, *2a*). Since only a small fraction of transformed human B lymphocytes permits viral replication, and since there is no fully permissive cell, it has been difficult to analyze the viral replicative cycle or to derive viral mutants. Until now the principal way to propagate the virus was to "immortalize" marmoset cells, a process that requires several weeks to months. Virus can then be harvested from supernatant fluids of continuous marmoset lymphoid cells which are more permissive of viral replication than comparable human cells (*3*, *4*).

The tropism of EBV for lymphocytes is apparently related at least in part to a virus "receptor" present on B cells (*5*). The host range of EBV can be enlarged if the usual mechanism of entry by the virus is bypassed (*6*). Microinjection of the DNA of the cytolytic EBV variant, P3HR-1, leads to viral "early antigen" expression in human and rat fibroblasts and monkey kidney cells. Implantation of B lymphocyte membranes by the process of membrane fusion has recently been shown to allow EBV to enter murine cells and T lymphocytes; the genome is expressed in such cells because they contain viral encoded antigens (*6*). Thus far, the only nonlymphoid cells in which infectious EBV has been propagated in the laboratory are malignant epithelial cells of nasopharyngeal carcinoma that have been passaged in nude mice (*4*). There has been no evidence that mature virus will replicate in a monolayer culture of uninfected normal fibroblastic cells, or that the viral DNA is infectious when conventional transfection methods are used.

For the experiments described here we chose human placental cells because we had found (*7*) that they were susceptible to transfection with the DNA's of

0036-8075/81/0424-0452$00.50/0

herpes simplex virus, BK virus, and SV40. Furthermore, human placental cells are effective feeder cells for EBV-transformed neonatal human lymphocytes, and increase plating efficiency by a factor of 10^2 (*8*). In our first experiments, the placental cells were transfected with EBV DNA; 4 hours later they were cocultivated with human umbilical cord leukocytes. In six consecutive experiments continuous lymphoblastoid cell lines appeared from 29 to 55 days after exposure of the placental cells to 1 μg of EBV (FF41) DNA. Cell lines failed to form when the transfecting mixture contained 100 ng or 10 ng of viral DNA or when only calf thymus DNA was present. Two different batches of FF41 DNA were infectious by this assay; in one experiment DNA from the B95-8 strain also proved infectious (Table 1). Incubation of FF41 DNA with the restriction endonucleases Sal I, Eco RI, Bam HI, Hind III, or Xba I eliminated transformation.

Lymphoblastoid cell lines that were derived in the transfection system, like lines established in parallel by exposure to intact virus, contained the EBV genome and expressed markers characteristic of B cells (Table 1). All the cells in every line were positive for EBV nuclear antigen; the lines lacked viral capsid antigen. There were between one and three EBV DNA copies per cell in lines FF366-3 and FF407-219, determined by the nucleic acid spot hybridization method (*9*). Neither of these two lines spontaneously released infectious EBV, nor could virus be recovered by the x-ray cocultivation technique (*10*). All the lines established in the transfection system had receptors for complement and contained surface and cytoplasmic immunoglobulins (Table 1). Only the μ class of immunoglobulin heavy chains was present, but both λ and κ light chains were found. Four lines that expressed only one type of light chain may be derived from a single transformed cell. Three lines contained both light chains and are thus a mixture of cells representing several initial transformation events.

At this point the data suggested several hypotheses to explain the origin of the lymphoblastoid cell lines. One was that the lymphocytes had been directly transfected by EBV DNA scavenged from the surface of the placental cells where DNA had remained adherent. Another hypothesis was that EBV DNA replicated in the cultured placental cells and produced mature virus that was then transferred to the cocultivated lymphocytes. Such virus might be transferred to the lymphocytes from the placental cells in cell-free form or by means of cell-to-cell contact or cell fusion. In favor of the idea that virus was responsible for the formation of the lymphoid lines in the cocultivation system was the observation that all the established lymphocyte lines were B cells, and thus mirrored the tropism of intact virus. Subsequent work demonstrated that virus replicated in the placental cells exposed to EBV DNA.

That the placental cells were required for successful transfection was evident from our inability to establish lymphoid

Table 1. Immunoglobulin synthesis by lymphoblastoid cell lines derived by cocultivation of HUCL and human placental cells exposed to EBV DNA. The EBV DNA was prepared from virions harvested from the medium of the B95-8 and FF41 marmoset lines (*12*). We isolated FF41 DNA using polyethylene glycol to concentrate the virus, and deoxyribonuclease to digest cellular DNA outside the virions. Virions were disrupted in 1 percent sarkosyl and proteinase K and the viral DNA was isolated by separation in two consecutive CsCl equilibrium gradients. The DNA was located in the gradients by spotting portions on DEAE paper (diethylaminoethyl cellulose) and staining with ethidium bromide (*13*). To test for the infectivity of EBV DNA we used the calcium technique of Graham and van der Eb (*14*). The EBV DNA (1 μg) was added to Hepes-buffered saline (HeBS; NaCl, 0.2*M*; KCl, 0.05*M*; Na_2HPO_4, 0.007*M*; dextrose, 0.055*M*; Hepes, 0.21*M*), *p*H 7.05, containing 125 m*M* $CaCl_2$, and 5 μg of calf thymus DNA in a 500-μl volume. The mixture was held at room temperature for 30 minutes and then added to a 1- to 6-day-old monolayer of a strain of cultured human placental cells in a 60-mm petri dish (approximately 10^6 cells per dish). The placental cell strain FF260 was used in passages 10 through 12. After 4 hours at 37°C, the placental cells were washed with Eagle's minimum essential medium (MEME) and treated with 25 percent glucose in HeBS for 4 minutes; then the monolayers were washed once with MEME containing 10 percent fetal bovine serum. Mixed mononuclear leukocytes from umbilical cord blood were prepared 1 to 6 days previously by Ficoll hypaque separation, and cultured in RPMI-1640 medium with 20 percent fetal calf serum. They were resuspended in MEME with 10 percent fetal calf serum and added to the transfected human placental cells at a concentration of 2×10^6 per milliliter. The cocultures were incubated in a CO_2 atmosphere for 7 to 10 days at which time the leukocytes were removed and placed in a flask containing either a passage of the human placental cells originally transfected or fresh, untreated placental cells. Controls, consisting of placental cells mock-transfected with a mixture lacking EBV DNA were always included. When cell lines had been established they were thereafter passaged free of the placental cell feeder layer. They were examined for EBV nuclear antigen by an immunofluorescent antibody technique with the addition of complement. Lymphocytes were tested for surface markers by rosette formation with sheep erythrocytes coated with antibody and complement. The presence of surface and intracytoplasmic immunoglobulin was detected by indirect immunofluorescence with specific antibodies directed against individual human heavy and light chains, purchased from Dako-Accurate.

Cell line	Transforming agent		Immunoglobulin					
			Surface			Cytoplasmic		
	Strain	Virus or DNA	μ	κ	λ	μ	κ	λ
FF 366-3	FF41	DNA	30	0	10	10	0	5
FF 407-219	FF41	DNA	41	0	30	7	0	6
FF 407-LS	B95	DNA	90	90	0	15	10	0
FF 407-323	FF41	Virus	90	85	0	21	5	0
FF 433-219	FF41	DNA	80	75	2	2	2	2
FF 433-303	FF41	DNA	60	42	14	21	11	9
FF 433-323	FF41	Virus	90	90	0	0	0	0
FF 444-219	FF41	DNA	76	50	20	14	15	9
FF 444-303	FF41	DNA	55	20	0	<1	0	0

Table 2. Demonstration of transforming activity in supernatant fluids from human placental cell cultures transfected with EBV DNA. Human placental cells were transfected with 1 μg of EBV (FF41) DNA under conditions described in the legend for Table 1. A portion of medium was removed daily from the culture dish and 0.1 ml of portion was tested without further centrifugation or filtration for transforming activity on a 1.0-ml culture of human lymphocytes (trial 1). Therefore, the assay cells were of increasing age in vitro as the experiment progressed. The remainder of the medium was stored frozen at −70°C and portions from day 1 through day 50 were tested on a second batch of neonatal human lymphocytes aged 1 day in vitro (trial 2). In trial 1, lymphocyte transformation was first noted 59 days after addition of fluids; in trial 2, transformation was evident after 20 days.

Day fluid harvested	Transforming activity present	
	Trial 1	Trial 2
1	−	−
2	+	−
4	+	+
5	+	+
6	+	+
7	−	+
8	−	+
10	+	+
12	+	+
15	−	+
16	−	+
17	−	−
24	−	−
50	−	−

lines from leukocytes exposed to the same batches and amounts of EBV DNA and cultured thereafter in the absence of feeder cells. However, since primary umbilical cord leukocytes are readily transformed in the absence of a feeder system when the transforming agent is virus rather than DNA, we performed the following experiment. We added EBV DNA to a culture dish of human placental cells and periodically removed a portion of the supernatant fluid to test for transforming activity on umbilical cord leukocytes without placental feeder cells (Table 2). In two trials, transforming activity was initially absent and became evident 2 days after exposure of placental cells to EBV DNA. Transforming activity continued to be detected at least up to 16 days after exposure of placental cells to EBV (FF41) DNA.

This experiment suggested that EBV replicated in the placental cells; therefore, we tested the placental cells transfected with EBV DNA for viral antigens. Both early antigen and viral capsid antigen were seen (Fig. 1) (*2a, 11*). No positive cells were observed when the smears were reacted with human serum lacking EBV antibody, nor were antigen-positive cells seen in placental cells treated only with carrier calf thymus DNA. Incubation of EBV DNA with deoxyribonuclease (20 μg/ml) destroyed its ability to induce antigens. Estimates of the efficiency of antigen induction following transfection with EBV DNA are shown in Table 3.

To test the notion that human placental cells are susceptible to intact virus, we inoculated such cells with 10^5 infectious units of either FF41 or HR-1 EBV. Virus was added directly in medium as well as under conditions used for transfection with DNA. No cells with antigen were observed.

We have not observed focus formation or other cytopathic effects in placental cells transfected with EBV DNA, nor have we been able to demonstrate "immortalization" of the placental cells.

The results provide evidence that EBV is able to undergo a full replicative cycle in a nonlymphoid cell in culture, provided that the barrier to infection of that cell is first overcome by transfection with DNA. Human placental cells are sensitive to DNA of the cytolytic and transforming strains and, in the case of the latter, progeny virus with intact transforming ability is produced. The exact nature and origin of the cell in these cultures from human placenta which supports EBV replication remains to be defined. That these cells do not themselves seem to become transformed or immortalized suggests that EBV genes which confer such properties onto B lymphocytes might not, for a variety of reasons, be operative in other cell types. Other cells may not respond to these viral functions in the same way or may not inhibit expression of certain EBV genes that are incompatible with lymphocyte immortalization. The availability of an assay for infectious EBV DNA should enable investigators to begin to assign biologic functions to various regions of the genome.

Table 3. Estimated efficiency of induction of early antigen, viral capsid antigen, and transforming virus in human placental cells exposed to EBV DNA. The conditions of transfection are described in the legend for Table 1. Human placental cells were exposed to serial dilutions that contained between 1 μg and 1 ng of DNA from each of two strains. After 96 hours about 30,000 cells were spread with a cytocentrifuge and examined for early antigen and viral capsid antigen by indirect immunofluorescence with human antiserums. The antigen-inducing titer is calculated from the lowest DNA dilution that gave a positive result and is corrected for the total number of cells that were exposed to DNA.

Strain	Early antigen-inducing units per microgram of DNA	Viral capsid antigen-inducing units per microgram of DNA	Lymphocyte-transforming units per microgram of DNA
HR-1	6×10^3	9×10^2	Negative
FF41	1.2×10^3	1×10^3	< 10

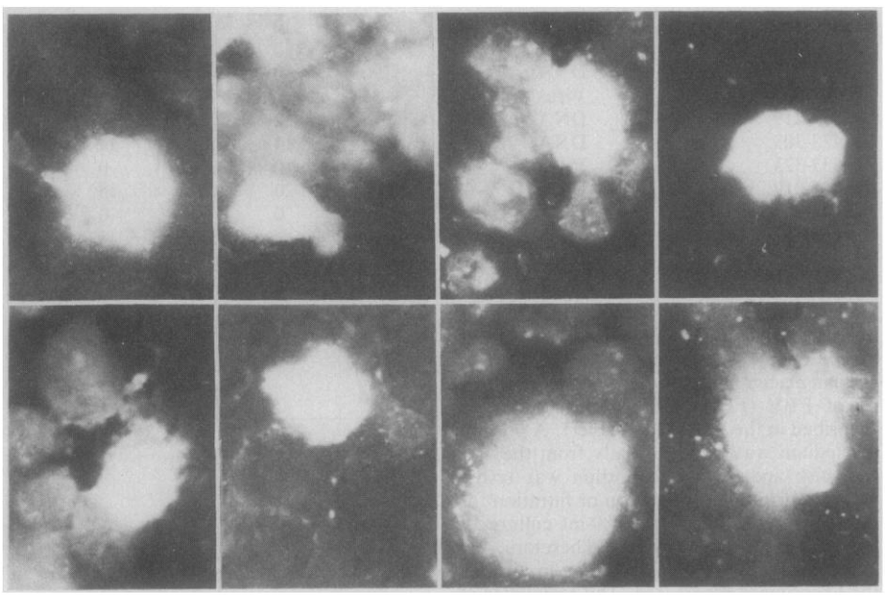

Fig. 1. Early antigen expression in cultured human placental cells exposed to EBV DNA. Human placental cells in a 60-mm petri dish were exposed to EBV (FF41) DNA in a $CaPO_4$ precipitate, as described in the legend for Table 1. After 4 days of incubation at 37°C the cells were dispersed with a mixture of trypsin and EDTA, and the cell spreads prepared with a cytocentrifuge were fixed in acetone. They were tested for early antigen with a pool of human antibody to EBV (dilution 1:320) obtained from two patients with chronic lymphocytic leukemia.

GEORGE MILLER
ELIZABETH GROGAN, LEE HESTON
JAMES ROBINSON*, DOUGLAS SMITH
Departments of Pediatrics and
Epidemiology and Public Health,
Yale University School of Medicine,
New Haven, Connecticut 06510

References and Notes

1. W. Henle, V. Diehl, G. Kohn, H. zur Hausen, G. Henle, *Science* **157**, 1064 (1967); J. H. Pope, M. D. Horne, W. Scott, *Int. J. Cancer* **3**, 857 (1968); P. Gerber, J. Whang-Peng, J. H. Monroe, *Proc. Natl. Acad. Sci. U.S.A.* **63**, 740 (1969); G. Miller, J. F. Enders, H. Lisco, H. I. Kohn, *Proc. Soc. Exp. Biol. Med.* **132**, 247 (1969); G. Miller, T. Shope, H. Lisco, D. Stitt, M. Lipman, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 383 (1972).
2. Y. Hinuma, M. Kohn, J. Yamaguchi, D. J. Wudarski, J. R. Blakeslee, J. T. Grace, Jr., *J. Virol.* **1**, 1045 (1967); G. Miller, J. Robinson, L. Heston, M. Lipman, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4006 (1974); J. Menezes, W. Leibold, G. Klein, *Exp. Cell Res.* **92**, 478 (1975).
2a. W. Henle, G.. Henle, B. A. Zajac, G. Pearson, R. Waubke, M. Scriba, *Science* **169**, 188 (1970).
3. G. Miller and M. Lipman, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 190 (1973).
4. D. H. Crawford, M. A. Epstein, G. W. Bornkamm, B. G. Achong, S. Finerty, J. L. Thompson, *Int. J. Cancer* **24**, 294 (1979).
5. M. Jondal and G. Klein, *J. Exp. Med.* **138**, 1365 (1973); ———, M. B. A. Oldstone, V. Bokish, E. Yefenof, *Scand. J. Immunol.* **5**, 401 (1976).
6. A. Graessmann, H. Wolf, G. W. Bornkamm, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 433 (1980); D. J. Volsky, I. M. Shapiro, G. Klein, *ibid.*, p. 5453.
7. G. Miller, E. Grogan, L. Heston, J. Robinson, T. Smith, unpublished data.
8. E. A. Grogan, J. F. Enders, G. Miller, *J. Virol.* **5**, 406 (1970); E. Henderson, G. Miller, J. Robin-

son, L. Heston, *Virology* **76**, 152 (1977). Thus far cells from only one placental cell strain have been tested as recipients of DNA. However, viral antigens have also been observed in human foreskin fibroblast cultures treated with EBV DNA.
9. J. Brandsma and G. Miller, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6851 (1980).
10. G. Wilson and G. Miller, *Virology* **95**, 351 (1979).
11. G. Henle and W. Henle, *J. Bacteriol.* **91**, 1248 (1966).
12. FF41 is a marmoset lymphoblastoid line that was transformed by an EBV isolated from the saliva of a patient with uncomplicated infectious mononucleosis. By comparison with B95-8, the genome of FF41 is not defective and contains approximately 8×10^6 megadaltons of extra genetic information located in the region of the Eco RI "C," Bam HI "I," and Hind III "D" fragments of B95-8 (G. Miller, D. K. Fischer, L. Gradoville, L. Heston, M. W. Weststrate, W. Maris, J. Brandsma, in preparation).
13. A. Adams, *J. Gen. Virol.* **20**, 391 (1973); B. Sugden, M. Phelps, J. Domoradzki, *J. Virol.* **31**, 590 (1979).
14. F. L. Graham and A. J. van der Eb, *Virology* **52**, 456 (1973).
15. The B95-8 DNA was supplied by Life Sciences, Inc., through its office of Biologic Resources, National Cancer Institute. Supported by PHS grants CA 12055, CA 16038, and AI 14741, and by grant VC-107 from the American Cancer Society.
* J.R. is a scholar of the Leukemia Society of America

25 September 1980; revised 21 November 1980

Suppressible Four-Base Glycine and Proline Codons in Yeast

Abstract. *Five ICR-170–induced mutations at the* His*4 locus in yeast are +1 G·C (G, guanine; C, cytosine) additions in DNA regions that contain multiple G·C base pairs. These mutations represent both nonsuppressible and suppressible alleles. All externally, suppressible frameshift mutations occur in glycine and proline codons to produce the four-base codons GGGU (U, uracil), GGGG, and CCCU. This implies that suppression of these four-base codons in yeast, as in bacteria, involves a four-base anticodon or its functional equivalent. Two identical four-base codons (CCCU) at widely separate regions within* His*4 are not suppressed equally.*

Frameshift mutations result from the addition or deletion of bases to the DNA (*1*). The most complete information on frameshift mutations comes from studies on enteric bacteria (*2*) and their bacteriophages (*3*). In these systems, planar aromatic hydrocarbons have been shown to induce frameshift mutations in regions of the DNA that contain sequences of repeated bases. Reversion of frameshift mutations can occur by a second, compensating frameshift within the same gene, or by a suppressor mutation in a different gene. A class of compounds, the ICR series (*4*), induces frameshift mutations at several different loci in *Salmonella typhimurium* and *Escherichia coli* (*2, 5*). Mutations caused by one of these compounds, ICR-191, have been most extensively characterized in the *hisD* gene of *S. typhimurium*. In this gene, ICR-191 causes base additions or deletions in monotonous runs of G·C base pairs (G, guanine; C, cytosine) (*6*). Two major groups of ICR-191–induced, suppressible mutations have been characterized. One group of mutations generates the four-base codon GGGG (code for glycine) (*7*) and the other group generates the four-base codon CCC· (proline) (*8*). In most instances the suppression results, respectively, from an alteration in a glycine or a proline transfer RNA (tRNA) (*9*, *10*). The suppressor mutation at *sufD* has been shown conclusively to involve the mutation of the anticodon of the glycl-tRNA from CCC → CCCC (*9*). Suppression by *sufD* is presumed to involve recognition of a four-base glycine codon by the mutant tRNA.

ICR-170 (*11*) is a powerful mutagen in eukaryotes including *Drosophila*, ascites tumor cells, yeast, and other fungal organisms (*4*, *12*). Mutations at the *his*4 locus of *S. cerevisiae* (*13*) are similar to those induced by ICR-191 in bacteria. Three groups of ICR-170–induced *his*4 mutations were identified on the basis of reversion and suppression analysis. Group I mutations failed to revert with ICR-170 and were not suppressed by external suppressors. Group II and group III mutations revert at high frequency with ICR-170 and are suppressed by external suppressors. Suppressors of group II mutations fail to suppress group III mutations, and similarly suppressors of group III mutations fail to suppress group II mutations. Nineteen ICR-170–induced *his*4 mutations are group II and two are group III. One of the suppressors of group II frameshift mutations alters the chromatographic behavior of a glycyl-tRNA species (*13*). These early studies on the ICR-170–induced mutations in yeast led to the suggestion that ICR-170 was making frameshift mutations in runs of G or C and that the ICR-170–induced suppressors were acting at the level of translation to suppress a four-base code word.

We now report the DNA sequence of five ICR-170–induced mutations at the *his*4 locus in yeast. One of the mutations, *his*4-506, is representative of the nonsuppressible group I mutations. Four of the mutations, two from group II, *his*4-38 and *his*4-519, and two from group III, *his*4-712 and *his*4-713, are representative of the two mutually exclusive classes of suppressible mutations. The DNA sequence data show conclusively that ICR-170 causes frameshift mutations in G·C regions of yeast DNA.

ICR-170–induced mutations at the *his*4 locus were cloned by the "integration and excision" method (*14*). Haploid yeast strains were genetically constructed to contain the ICR-170–induced mutation, as well as the *ura*3 mutation *ura*3-52. Strains were transformed with a pBR322 plasmid containing a functional *ura*3 gene and the proximal Eco RI restriction fragment from the *his*4 locus (*15*), selecting for the Ura$^+$ phenotype.

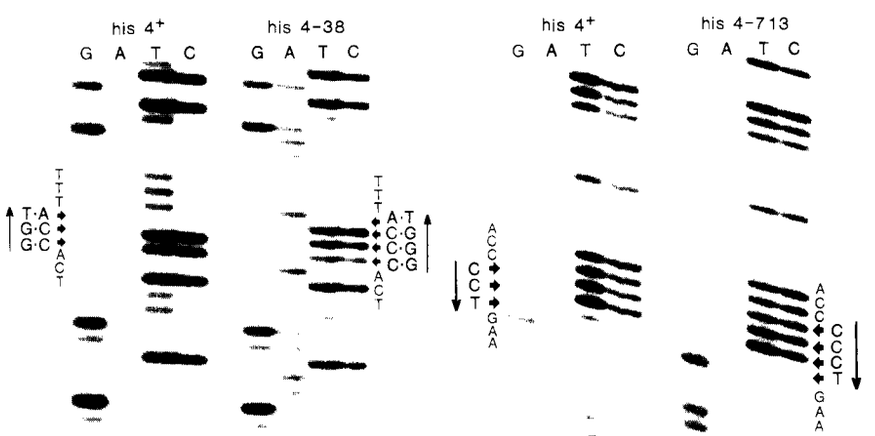

Fig. 1. The DNA sequence of a suppressible glycine and proline four-base codon. The autoradiograms of the DNA sequences of frameshift mutations *his*4-38 and *his*4-713 are presented in comparison to the DNA sequence from the corresponding wild-type region. The antisense strand of *his*4-38 was sequenced and is presented, base-paired, to emphasize the suppressible four-base codon. (GGGT (T, thymine); *his*4-713 is the suppressible four-base codon CCCT. Note the +1 addition, as well as the +1 shift in the sequence ladder.

0036-8075/81/0424-0455$00.50/0