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Human T-cell leukemia virus type II transforms normal human lymphocytes

(human retrovirus/hairy-cell leukemia/T-cell lines/lymphokines)

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ABSTRACT **A unique human retrovirus (human T-cell leukemia virus type II, HTLV-II), isolated from a patient with a T-cell variant of hairy-cell leukemia, has been shown to be distinct from the more common isolates of human T-cell leukemia virus. This virus was tested for its ability to transform normal human peripheral blood lymphocytes. The HTLV-II-infected T-cell line Mo-T was lethally x-irradiated and cocultivated with normal human peripheral blood lymphocytes. The cocultivation of normal cells with Mo-T cells resulted in the transformation of the normal cells as evidenced by the establishment of permanent cell lines. The transformed cells are infected with HTLV-II as shown by immunologic tests and molecular hybridization. The cells are of mature T-cell phenotype and constitutively produce lymphokines. An Epstein–Barr virus-transformed lymphoblast B-cell line established from peripheral blood cells of the patient Mo, designated Mo-B, also was found to be infected with HTLV-II. All HTLV-II-infected cells, including the Mo-B cells, were capable of transforming normal cells of T-cell phenotype by transmission of virus by cocultivation. These results indicate that HTLV-II infects both B and T cells but transforms normal human peripheral blood lymphocytes of T-cell phenotype.**

Closely related retroviruses (1) designated as human T-cell leukemia virus type I (HTLV-I) or adult T-cell leukemia virus (ATLV) are associated with aggressive malignancies of mature T cells, including T-cell lymphosarcoma cell leukemia of West Indian Blacks (2), Japanese adult T-cell leukemia (3), and sporadic T-cell leukemias elsewhere (4). These viruses have been shown to be capable of transforming human lymphocytes by cocultivation of normal cells with virus-infected cells from patients (5–7).

Recently, a human retrovirus designated HTLV type II (8) was identified in a patient with a leukemia different from typical HTLV-associated disease (9). This patient had a T-cell variant of hairy-cell leukemia and is alive and well 7 years after undergoing a splenectomy. A cell line (Mo-T) was derived from the patient's spleen (10). The Mo-T cell line is of mature T-cell phenotype and produces a number of different lymphokines. This cell line was later shown to be infected with HTLV-II. HTLV-II is distinct from the more common isolates of HTLV or ATLV by immunologic (8) and molecular criteria (11). Because of genetic differences between HTLV-I and HTLV-II and the relatively benign disease with which HTLV-II was associated, we investigated whether HTLV-II also could transform normal human cells. We report here that normal human peripheral blood cells can be rapidly transformed by cocultivation with HTLV-II-infected T and B cells. The transformed cells are of mature T-cell phenotype and constitutively produce lymphokines similar to the Mo-T cell line (12–14).

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METHODS

Cells and Viruses. All cells were propagated in Iscove's medium supplemented with penicillin, streptomycin, and fetal bovine serum. The HTLV-II-infected Mo-T cells have been described (8, 10).

The Mo-B cell line was established from the patient, Mo, about 2 years after splenectomy by using Ficoll/Hypaque-separated peripheral blood mononuclear cells. The cells were maintained in suspension culture without further treatment or additives.

The HTLV-I-infected cell line, ME, was derived from a patient with typical HTLV-I-associated disease (15, 16). The ME cells are T lymphoblasts, which express viral p19 and p24 antigens by immunofluorescence assays, and are capable of transforming normal human peripheral blood cells by cocultivation (16).

Infection of cells by virus was assayed by indirect immunofluorescence with a monoclonal antibody directed against the viral p19 antigens (17) or a goat antibody against purified viral p24 antigens (4). The presence of viral reverse transcriptase was assayed as described (18) after centrifugation of virions from 30 ml of supernatant medium.

Transformation of Human Peripheral Blood Cells. Mo-T cells (5×10^5) irradiated with 10,000 rads were mixed with an equal number of low-density Ficoll/Hypaque-separated peripheral blood mononuclear cells at a final concentration of 10^6 cells per ml in Iscove's medium supplemented with 20% fetal bovine serum. Few peripheral blood cells stained positively for p19 or p24 antigens during the first week of coculture. (The irradiated Mo-T cells were distinguished from peripheral blood cells by their greater size.) By the second week of coculture, p19- and p24-positive cells were evident, appearing primarily as small clusters. In most cultures these cells continued to grow with a doubling time of approximately 48 hr.

Assays of Cell Surface Phenotype. The presence of surface membrane immunoglobulin was determined by immunofluorescence. Erythrocyte-rosette-forming cells were determined with sheep erythrocytes. All OKT (Ortho Instruments) surface phenotypes were determined by indirect immunofluorescence with a fluorescence-activated cell sorter.

Assays of Lymphokine Production. Conditioned medium was harvested from cells given fresh medium 4 days previously. Colony-stimulating factor (CSF) was assayed by using Ficoll/Hypaque-separated, light-density, nonadherent bone marrow cells as described (12). Erythroid-potentiating activity (EPA) was assayed with peripheral blood buffy coat cells (13) by determining an increment in the number of large erythroid clones (BFU-E) formed. Neutrophil migration-inhibitory factor (NIF)

Abbreviations: ATLV, adult T-cell leukemia virus; EBV, Epstein–Barr virus; HTLV, human T-cell leukemia virus; kbp, kilobase pair(s).

activity was assayed by measuring the inhibition of migration of purified neutrophils in agarose (14).

DNA Extraction, Molecular Hybridization, and Source of Hybridization Probes. Total cellular DNA was extracted from cells by using proteinase K digestion, phenol extraction, and ribonuclease A digestion. The HTLV-II cDNA clones, pH-13 and pH-3, are described in more detail elsewhere (11). Briefly, they were obtained by screening a cDNA library prepared from poly(A)-containing RNA extracted from Mo-T cells. The cDNA was prepared by standard methods (19) and inserted into the *Pst* I site of pBR322. The cDNA clones were propagated in the bacterial host MC1061. The viral-specific cDNA clones were isolated by "colony hybridization" (20) with a hybridization probe synthesized from detergent-disrupted virions. The Epstein–Barr virus (EBV)-specific hybridization probe is specific for the large internal repeat (*Bam* W fragment) of the EBV genome (B. Sugden, University of Wisconsin, personal communication). The hybridization probes were labeled with ^{32}P by nick-translation (21). All hybridization procedures were by the method of Southern (22).

RESULTS

Transformation of Normal Human Peripheral Blood Cells. Infection of cells by cocultivation of infected cells with uninfected cells has been used with EBV (23) and ATLV (6) and HTLV-I (7). We adapted this procedure by using the Mo-T cell line as a source of HTLV-II for cocultivation with normal human peripheral blood cells. Transformation of peripheral blood cells was determined by the outgrowth of continuously growing cells from the mixed cultures. Because the Mo cells were originally derived from a male patient, female blood donors were used to confirm the origin of the resulting transformed cells. The Mo-T cells were irradiated with 10,000 rads to prevent their further proliferation.

Peripheral blood cells from control cultures without Mo-T cells or cocultivated with other irradiated cell lines not infected with HTLV ceased active growth beyond the first 2 weeks of culture. Peripheral blood cells cocultivated with irradiated Mo-T cells proliferated rapidly during the first week and continued to grow in large clusters indefinitely. A majority of the cocultures with Mo-T cells (20/30) could be established into permanent cell lines. (Of the lines that we have continued to propagate, the cells have been growing continuously for more than 9 months.) Of cells from five female donors tested, all were capable of being transformed. Several of the cell lines were subjected to karyotype analysis and were shown to be of normal female karyotype, confirming that normal cells had been transformed. Cells from all transformant lines expressed p19 and p24 antigens and they all produced virus as determined by assays for viral reverse transcriptase activity in conditioned medium.

DNA was extracted from three of the transformed cell lines and analyzed for the presence of viral DNA sequences by molecular hybridization. A cDNA clone specific for HTLV-II was used as a hybridization probe. The DNA sequences of this clone are homologous to regions of the HTLV-II genome which, when digested with *Eco*RI, result in a characteristic DNA fragment of 3.7 kilobase pairs (kbp). The HTLV-II viral sequences were present in DNA from Mo-T and from the new HTLV-II-transformed cells and were absent in other neoplastic cells including a HTLV-I-infected cell line, ME (Fig. 1), indicating that the transformation was associated with HTLV-II infection.

Seven cell lines were further characterized for cell phenotype. Some cells from all of the cell lines formed rosettes with sheep erythrocytes (Table 1). No surface membrane immunoglobulin was detected. The T-cell phenotype was confirmed

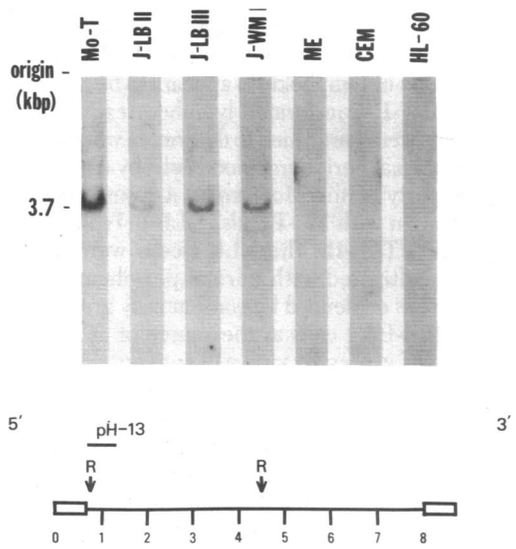

FIG. 1. Presence of HTLV-II-specific sequences in DNA of transformants derived by cocultivation with Mo-T cells. (*Upper*) DNAs from the various human cell lines were digested with restriction enzyme *Eco*RI and subjected to electrophoresis in agarose. The DNAs were hybridized with a ^{32}P-labeled HTLV-II cDNA clone (pH-13) by the method of Southern (22). J-LB II, J-LB III, and J-WM are HTLV-II transformants (Table 1). CEM is a human lymphoblast T-cell line. HL-60 is a human myeloid cell line. ME is a HTLV-I-infected lymphoid cell line. (*Lower*) Map of HTLV-II DNA (11). The positions of *Eco*RI (R) sites flanking the 3.7-kbp fragment are shown. The region of homology with pH-13 is indicated. The numbers indicate the size in kbp.

for most cell lines by the presence of immunofluorescence with the "pan T" monoclonal antibody OKT3. Studies with T-cell-specific monoclonal antibodies OKT4 and OKT8, distinguishing "helper-inducer" T cells and "suppressor" T cells, respectively, showed that all cell lines were of "helper-inducer" phenotype. This phenotype is similar to that of the Mo-T cell line and similar to transformants derived from HTLV-I or ATLV infection (6, 7, 16).

The transformed cells were also positive for cytochemical markers of T lymphocytes such as fluoride-resistant nonspecific esterase, β-glucuronidase, and acid phosphatase, as are the Mo-T cells (9, 10). A few cells from most lines were positive for tartrate-resistant acid phosphatase. They were negative for terminal deoxynucleotidyltransferase, a marker for immature lymphoid cells.

The Mo-T cells produce a number of lymphokines, including neutrophil migration-inhibitory factor (14), colony-stimulating factor (12), and erythroid-potentiating activity (13). All of the

Table 1. Surface phenotype of HTLV-II-transformed cell lines

Cell line	sIg$^+$ cells, %	E-rosette$^+$ cells, %	OKT3$^+$ cells, %	OKT4$^+$ cells, %	OKT8$^+$ cells, %
Mo-T	0	60	<1.0	93	<1.0
J-LB I	0	17	83	94	<1.0
J-LB II	0	38	64	87	<1.0
J-LB III	0	8	75	86	<1.0
J-LB IV	0	6	<1.0	89	<1.0
J-WM I	0	11	76	81	<1.0
J-WM(P)*	0	9	81	94	<1.0
J-KK I	0	41	81	81	<1.0

sIg, surface Ig; E-rosette, erythrocyte rosette.

* J-WM(P) was derived from a culture that was supplemented with 0.5% phytohemagglutinin (Wellcome). These cells are propagated in the continuous presence of phytohemagglutinin, although the absence of phytohemagglutinin has no apparent effect on cell growth.

transformant cell lines produced one or more of these lymphokines in amounts comparable to the Mo-T cells (Table 2). Thus, production of lymphokines appears to be a general property of all HTLV-II-transformed lymphocytes.

Experiments were performed to determine whether the ability to transform normal peripheral blood cells by cocultivation was a unique property of the Mo-T cells. A transformant derived from cocultivation with Mo-T cells, J-LB I (Table 1), was used as a source of HTLV-II. The J-LB I cells were lethally irradiated and cocultivated with normal peripheral blood cells. Transformation as evidenced by continuously growing cells was successful with J-LB I cells as the source of HTLV-II. These transformed cells expressed viral p19 antigens and were of mature T-cell phenotype. Transformation was successful when the source of the normal donor's cells was the same as the source of J-LB I cells (donor LB) (cells from six out of six cultures were transformed), and when the normal donor cells were different (donor AN) (cells from six out of six cultures were transformed), indicating that a "mixed lymphocyte reaction" was not necessary for infection and transformation by HTLV-II.

Subsequent experiments have shown that all other HTLV-II transformant cell lines tested have been capable of transforming normal human peripheral blood cells by cocultivation. As few as 50 irradiated HTLV-II-infected cells are sufficient to transform normal cells when cocultivated with 5×10^5 peripheral blood cells. However, similar to reports by other investigators working with ATLV (6), we were unable to transform normal peripheral blood cells by direct infection with cell-free supernatants or concentrated virus (unpublished observation).

We established a lymphoblast B-cell line (Mo-B) from the patient Mo about 2 years after establishment of the Mo-T cell line. This B-cell line has surface membrane immunoglobulin, does not rosette with sheep erythrocytes, does not react with OKT3, OKT4, OKT8, or OKT11 T-cell-specific monoclonal antibodies with a fluorescence-activated cell sorter, and does not produce the lymphokines colony-stimulating factor, neutrophil migration-inhibitory factor, or erythroid-potentiating activity. This B-cell line was likely to be transformed by EBV because the EBV capsid antigen (VCA) and EBV nuclear antigen (EBNA) were detected by immunofluorescence, and molecular hybridization demonstrated the presence of the EBV genome (Fig. 2). This cell line also was found to be infected with HTLV-II. Viral antigens p19 and p24 were detected by immunofluorescence, and molecular hybridization demonstrated the presence of the HTLV-II genome (Fig. 2).

When the Mo-B cell line was irradiated and used in cocultivation studies with normal peripheral blood cells, transformed cultures were established that showed a phenotype similar to that of other HTLV-II-transformed cell lines, namely, T-cell phenotype and the production of lymphokines. These transformed cells expressed viral p19 and p24 antigens, and the HTLV-II genome was present (data not shown). The EBV genome was not present in any of the transformed cells (data not shown).

Table 2. Lymphokine production by HTLV-II-transformed cell lines*

Cell line	Activity of lymphokines		
	CSF	EPA	NIF
Mo-T	+	+	+
J-LB I	+	+	++
J-LB II	+	NT	+
J-LB III	+	NT	++
J-LB IV	+	NT	+
J-WM I	+	+	+
J-WM(P)	+	±	++
J-KK I	+	±	+

CSF, colony-stimulating factor; EPA, erythroid-potentiating activity; NIF, neutrophil migration-inhibitory factor; NT, not tested.
*The amounts of activity produced relative to the Mo-T cell line are shown for one representative experiment. The relative amounts of activity varied with passage of the cells.

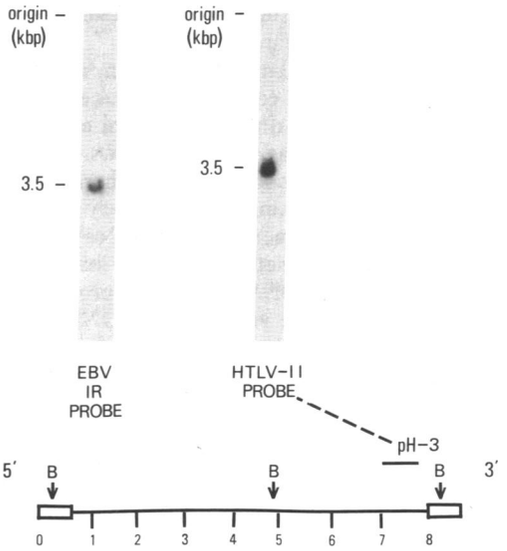

FIG. 2. Presence of EBV- and HTLV-II-specific sequences in the Mo-B cell line. DNA from the Mo-B cell line was digested with restriction enzyme *Bam*HI and subjected to electrophoresis in agarose (*Upper*). The DNAs were hybridized with either a probe specific for the large internal repeats of EBV (EBV IR probe is homologous to repeated 3.5-kbp *Bam*HI fragments) or a HTLV-II-specific cDNA probe (pH-3), the position of which is indicated on the map of the HTLV-II genome (11) (*Lower*). The *Bam*HI sites (B) are indicated on the HTLV-II genome. The numbers indicate the size in kbp. The region between the *Bam*HI sites at 4.7 and 8.2 kbp represents the 3.5-kbp fragment detected with pH-3. The two lanes shown are from different agarose gels, and the autoradiograph exposures are not comparable.

We also successfully infected an EBV-transformed B-cell line (obtained from peripheral blood of a healthy individual) by cocultivation with lethally irradiated Mo cells. After infection, viral antigens continued to be expressed for several months. This HTLV-II-infected B-cell line could be cocultivated with normal peripheral blood cells resulting in transformed cells of T-cell phenotype.

These experiments demonstrate that the ability to transform normal cells by cocultivation is not a unique property of HTLV-II-infected T cells but also can be accomplished by transmission of HTLV-II from infected B cells. Therefore, these results provide evidence that HTLV-II itself is responsible for transforming the target cells in normal human peripheral blood.

DISCUSSION

Using cocultivation as a means of infecting normal human cells with HTLV-II, we demonstrated that virus infection is necessary for transformation of normal human lymphocytes. HTLV-II could be transmitted from both HTLV-II-transformed T cells and from HTLV-II-infected B cells. Because transformation could be accomplished by autologous cocultivation of a HTLV-II transformant with normal cells of the same blood donor, a histoincompatibility-stimulated mitogenic response appears not to be necessary for transformation. Because HTLV-II also can be transmitted from a B-cell line that does not produce lymphokines, T-cell lymphokine-induced mitogenic responses do not appear to be necessary for transformation. Therefore, it is likely

that HTLV-II infection is directly responsible for the continued proliferation of the transformed cells.

The transformed cells have a mature T-cell phenotype and indefinite growth potential similar to cells transformed by ATLV and HTLV-I as reported by others (5–7). We also have tested a HTLV-I-infected T-cell line (ME) (16) and found that the efficiency of transformation was comparable to that using Mo-T. The two types of HTLV are distinguished by partial immunological crossreactivity (8), and the viral genomes are only distantly related by nucleic acid hybridization (11). HTLV-I is associated with aggressive highly malignant T-cell leukemia/lymphomas. By contrast, HTLV-II is associated with a relatively benign form of hairy-cell leukemia in the only known patient, Mo (9, 10). The ability of both types of HTLV to transform lymphocytes *in vitro* having similar phenotypes most likely reflects a common mechanism of action at the cellular level. The subsequent progression of these transformed cells to different neoplastic diseases in patients may reflect differences in the structure of the viruses. The role of HTLV-II in human malignancy, however, remains unclear because only one patient has been identified with a disease associated with this virus.

The Mo-T cell line is a source of many lymphokines, some of which have been extensively purified and characterized (12–14, 16). The production of lymphokines appears to be a general property of the HTLV-II-transformed cell lines. HTLV-I-transformed cell lines also produce lymphokines (15, 16, 24). The availability of new cell lines that produce lymphokines may provide additional tools for the study of lymphokine expression in "helper" T cells.

EBV-transformed B-cell lines that also are infected with ATLV were previously reported from patients with adult T-cell leukemia (25). The B-cell line isolated from the patient Mo appears to be similar to these cell lines as it is infected with both EBV and HTLV-II. The Mo-B cell line produces HTLV-II and is capable of transforming normal peripheral blood T cells. We also have been able to infect EBV-transformed B-cell lines *in vitro* with stable integration of the HTLV-II genome, although viral antigens may not always be expressed (unpublished data). Infection of B cells or their precursors may be important in the pathogenesis of HTLV-II-associated disease.

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1. Popovic, M., Reitz, M. S., Jr., Sarngadharan, M. G., Robert-Guroff, M., Kalyanaraman, V. S., Nakao, Y., Miyoshi, I., Minowada, J., Yoshida, M., Ito, Y. & Gallo, R. C. (1982) *Nature (London)* **300,** 63–66.
2. Blattner, W. A., Kalyanaraman, V. S., Robert-Guroff, M., Lister, T. A., Galton, D. A. G., Sarin, P. S., Crawford, M. H., Catovsky, D., Greaves, M. & Gallo, R. C. (1982) *Int. J. Cancer* **30,** 257–264.
3. Hinuma, Y., Nagata, K., Hanaoka, M., Nakai, M., Matsumoto, T., Kinoshita, K.-I., Shirakawa, S. & Miyoshi, I. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6476–6480.
4. Gallo, R. C., Kalyanaraman, V. S., Sarngadharan, M. G., Sliski, A., Vonderheid, E. C., Maeda, M., Nakao, Y., Yamada, K., Ito, Y., Gutensohn, N., Murphy, S., Bunn, P. A., Jr., Catovsky, D., Greaves, M. F., Blayney, D. W., Blattner, W., Jarrett, W. F. H., zur Hausen, H., Seligmann, M., Brouet, J. C., Haynes, B. F., Jegasothy, B. V., Jaffe, E., Cossman, J., Broder, S., Fisher, R. I., Golde, D. W. & Robert-Guroff, M. (1983) *Cancer Res.* **43,** 3892–3899.
5. Miyoshi, I., Kubonishi, I., Yoshimoto, S., Akagi, T., Ohtsuki, Y., Shiraishi, Y., Nagata, K. & Hinuma, Y. (1981) *Nature (London)* **294,** 770–771.
6. Yamamoto, N., Okada, M., Koyanagi, Y., Kannagi, M. & Hinuma, Y. (1982) *Science* **217,** 737–739.
7. Popovic, M., Sarin, P. S., Robert-Guroff, M., Kalyanaraman, V. S., Mann, D., Minowada, J. & Gallo, R. C. (1983) *Science* **219,** 856–859.
8. Kalyanaraman, V. S., Sarngadharan, M. G., Robert-Guroff, M., Miyoshi, I., Blayney, D., Golde, D. & Gallo, R. C. (1982) *Science* **218,** 571–573.
9. Saxon, A., Stevens, R. H. & Golde, D. W. (1978) *Ann. Intern. Med.* **88,** 323–326.
10. Saxon, A., Stevens, R. H., Quan, S. G. & Golde, D. W. (1978) *J. Immunol.* **120,** 777–782.
11. Chen, I. S. Y., McLaughlin, J., Gasson, J. C., Clark, S. C. & Golde, D. W. (1983) *Nature (London)* **305,** 502–505.
12. Golde, D. W., Bersch, N., Quan, S. G. & Lusis, A. J. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 593–596.
13. Lusis, A. J., Quon, D. H. & Golde, D. W. (1981) *Blood* **57,** 13–21.
14. Weisbart, R. H., Lusis, A. J., Kacena, A., Spolter, L., Eggena, P. & Golde, D. W. (1982) *Clin. Immunol. Immunopathol.* **22,** 408–418.
15. Koeffler, H. P. & Golde, D. W. (1983) *Clin. Res.* **31,** 410A (abstr.).
16. Gasson, J. C., Chen, I. S. Y., Westbrook, C. A. & Golde, D. W. (1983) in *Normal and Neoplastic Hematopoiesis,* UCLA Symposia on Molecular and Cellular Biology, New Series, eds. Golde, D. W. & Marks, P. A. (Liss, New York), Vol. 9, in press.
17. Robert-Guroff, M., Ruscetti, F. W., Posner, L. E., Poiesz, B. J. & Gallo, R. C. (1981) *J. Exp. Med.* **154,** 1957–1964.
18. Yoshida, M., Miyoshi, I. & Hinuma, Y. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2031–2035.
19. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
20. Grunstein, M. & Hogness, D. S. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 3961–3965.
21. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113,** 237–251.
22. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
23. Henle, W., Diehl, V., Kohn, G., zur Hausen, H. & Henle, G. (1967) *Science* **157,** 1064–1065.
24. Tarella, C., Ruscetti, F. W., Poiesz, B. J., Woods, A. & Gallo, R. C. (1982) *Blood* **59,** 1330–1336.
25. Yamamoto, N., Matsumoto, T., Koyanagi, Y., Tanaka, Y. & Hinuma, Y. (1982) *Nature (London)* **299,** 367–369.