Assay for Epstein-Barr Virus Based on Stimulation of DNA Synthesis in Mixed Leukocytes from Human Umbilical Cord Blood

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Received for publication 9 December 1974

Relationships between the rate of DNA synthesis in cultured human umbilical cord leukocytes and the multiplicity of added Epstein-Barr virus (EBV) were studied. At low multiplicities of approximately 0.1 transforming units/cell (approximately 10 physical particles/cell), inoculated cultures demonstrated increased rates of DNA synthesis, by comparison to uninoculated cultures, 3 days after inoculation. Stimulation of DNA synthesis was evident at progressively longer intervals after inoculations of 10-fold dilutions of virus. The rate of DNA synthesis, determined by short [^3H]thymidine pulses, reflected as small as twofold changes in multiplicity and thus can serve as a quantitative assay for the virus. Changes in the rate of DNA synthesis were evident before increases in cell number or alteration in morphology. Stimulation of DNA synthesis in umbilical cord leukocytes was inhibited by treatment of EBV with antibody and also in graded fashion, by progressive doses of UV irradiation to the virus. Induction of DNA synthesis by EBV was serum dependent. Estimates of the number of cells transformed were obtained by extrapolation from a standard curve relating known numbers of transformed cells to [^3H]thymidine incorporation and also by cloning cells after exposure to virus. At the low multiplicities of infection used in these experiments approximately 0.04 to 0.002 of the total cellular population was transformed. The high efficiency of cell transformation by EBV by comparison to other DNA tumor viruses is emphasized.

Some strains of Epstein-Barr virus (EBV) cause normal human leukocytes with a limited life span in vitro to proliferate indefinitely, forming continuous lymphoblastoid cell lines (8, 10, 15, 16, 18). This process, transformation or immortalization, is recognized by a change in morphology of infected cells to a lymphoblastoid appearance, clumping of cells, acid production, rapid increase in cell number, and acquisition by the culture of the ability to be repeatedly subdivided. The mechanism of this phenomenon has not been studied in detail principally because of the lack of quantitative methods.

Recent studies by Gerber and Hoyer (7) and Pope et al. (19), have shown that EBV stimulates the incorporation of [^3H]thymidine ([^3H]TdR) into acid insoluble material of infected cells. [^3H]TdR is incorporated into material with the density of cellular DNA (7). Thus, stimulation of host cell DNA synthesis may be an important early event in transformation. In the present investigation we sought to determine the relationship between the rate of [^3H]TdR incorporation by EBV-treated leuko-

cytes and the amount of virus added. These experiments had two general objectives: The first was to obtain data about the efficiency of DNA stimulation and transformation and the second was to ascertain whether [^3H]TdR incorporation might serve as the basis for a rapid, objective assay for biologically active virus.

MATERIALS AND METHODS

Preparation of virus stocks. EBV was prepared from marmoset lymphoblastoid cell line (B95-8) 7 days after the cells had been seeded at 3×10^4 cells/ml in medium RPMI 1640 plus 10% fetal calf serum and incubated at 36 C (13). Supernatant culture fluid was centrifuged to remove cells, frozen and thawed three times, and passed through a 0.8- μm membrane filter (Millipore Corp.). Infectivity of the stocks ranged from 4.0 to 5.0 $\log_{10}$ 50% transforming doses (TD_{50}) per 0.1 ml by the morphologic assay. In this assay replicate cultures of leukocytes from human umbilical cord blood are inoculated with serial 10-fold dilutions of virus and observed until an end point, the presence or absence of transformation observed microscopically is reached, usually after 40 days (13). The stocks contained approximately 10^4 to 10^7 phys-

ical particles/ml as determined by virus particle counts of negatively stained hanging drop preparations (14). Stocks, determined to be free of bacteria, fungi, and mycoplasma, were stored at -70°C .

Leukocyte culture. Two methods were used. (Method A) Leukocytes were separated from approximately 20 ml of heparinized umbilical cord blood by addition of 3 ml of 6% Dextran (molecular weight 240,000) in 0.15 M NaCl for 1 h. The leukocyte-rich supernatant was centrifuged at $200 \times g$ for 10 min, the pellet was washed three times in Puck saline A (PSA) (20), and the cells were resuspended in medium RPMI 1640 plus 20% fetal calf serum with penicillin (50 U/ml), streptomycin (50 $\mu\text{g}/\text{ml}$), and amphotericin B (1 $\mu\text{g}/\text{ml}$). The cell concentration was adjusted to 1.5 to 2.0×10^6 cells/ml. The leukocyte suspension was distributed to stoppered tubes (13 by 100 mm) in 0.4-ml aliquots, and 0.1 ml of virus stock or medium was added. A group of identical tubes were used for each parameter to be studied so that, at intervals after inoculation, a single tube from each group was harvested for determination of [^3H]Tdr incorporation. The cultures were incubated at 36°C in an atmosphere of 5% CO_2 and air. Medium was changed twice weekly. (Method B) This method employed a single culture vessel for each parameter and aliquots of 0.5 ml were periodically removed for assay of [^3H]Tdr incorporation. Leukocytes were separated from cord blood by dextran sedimentation or, in some cases, by a Ficoll-Hypaque gradient (3). In the latter case, cord blood was diluted 1:1 in PSA and layered over Ficoll-Hypaque. The gradient was centrifuged at $400 \times g$ at room temperature for 40 min. The leukocytes and some adjacent plasma at the interface were collected and centrifuged at $200 \times g$ for 10 min. Leukocytes were then washed three times with PSA and resuspended in medium. Ficoll-Hypaque separation resulted in a 95 to 99% pure mononuclear cell population.

Leukocyte cultures were established in stoppered 3-oz. prescription bottles at a cell concentration of 1 to $1.5 \times 10^6/\text{ml}$. For each 4 ml of cell suspension 1 ml of virus or, as a control, medium was added. The usual starting volume was 5 ml. The conditions of incubation were the same as for Method A. Medium of cultures in prescription bottles was partially changed twice weekly, usually 1 to 2 days before the [^3H]Tdr pulse.

[^3H]Tdr pulses. [^3H]Tdr incorporation into acid-insoluble material was measured in infected and uninfected cultures. For leukocytes cultured by method A the [^3H]Tdr concentration (specific activity 6.7 Ci/mM; New England Nuclear) was 5.0 $\mu\text{Ci}/\text{ml}$ in a volume of 2 ml of warm medium. After 30 min in a 37°C water bath, the reaction was stopped by the addition of cold PSA. The cells were washed again in cold PSA. The cell pellet was resuspended vigorously in 3 ml 5% trichloroacetic acid (wt/vol) in water and the tubes were placed on ice for a minimum of 20 min. The precipitates were collected on 2.5-cm membrane filters (Millipore Corp., type HA, 0.45 μm) and washed three times with cold 5% trichloroacetic acid. The filters were placed in vials, and dried, 5 ml of scintillation fluid containing toluene, Triton X-100,

and Liquifluor were added, and radioactivity was determined in a liquid scintillation counter.

For method B the pulse was performed on a 0.5-ml aliquot of cell suspension removed from the original culture bottle and placed in a tube (13 by 100 mm). The concentration of isotope was 6.25 $\mu\text{Ci}/\text{ml}$ in 1 ml and the duration of pulse was 1 h at 37°C .

UV-irradiation of EBV. One-milliliter aliquots of virus stock in spent culture medium were placed in 60-mm petri dishes. UV light was delivered by a 15-W GE 15T8 low-pressure mercury germicidal lamp. UV dose-rate was 10 ergs/ mm^2 per s determined by a dosimeter constructed and calibrated by R. Latarjet (11) and kindly loaned by D. Rupp.

RESULTS

Comparison of titration of EBV by [^3H]Tdr incorporation with titration by the morphologic assay. The kinetics of DNA synthesis were followed in leukocyte cultures inoculated with 1.0 ml of serial 10-fold dilutions (to 10^{-6}) of a virus stock (Fig. 1). Stimulation of [^3H]Tdr incorporation in infected cultures above the level for an uninoculated culture was evident within 3 days after exposure to undiluted virus. Uninfected cells showed a low level of spontaneous [^3H]Tdr uptake which lasted for 3 weeks and then declined. Each 10-fold dilution of virus to 10^{-5} caused eventual stimulation of [^3H]Tdr uptake; the 10^{-6} dilution did not cause stimulation, and the curve of [^3H]Tdr incorporation for this dilution resembled that of the uninfected culture. There was a direct relationship between the amount of virus added and the time before stimulation of [^3H]Tdr incorporation was evident. Results of two replicate experiments are shown in Fig. 2.

The rate of [^3H]Tdr uptake, once stimulation

FIG. 1. Rate of [^3H]Tdr incorporation in leukocyte cultures after exposure to serial 10-fold dilutions of EBV or, as control, medium. Leukocyte cultures and [^3H]Tdr pulses were performed according to method B. The multiplicity with undiluted virus was approximately 10 particles/cell.

FIG. 2. Relationship between titer of virus inoculum and time before stimulation of DNA synthesis was detected. Stimulation for purposes of this chart is considered $\geq 2,000$ counts/min above background of uninfected cultures. Data from two replicate experiments (one of which is illustrated in Fig. 1).

occurred, increased logarithmically to a maximum rate which was a permanent feature of the transformed cells as long as fresh medium was supplied. All cultures with evidence of stimulation of $[^3\text{H}]\text{Tdr}$ incorporation following addition of EBV eventually formed continuous lymphoblastoid cell lines. However, stimulation of incorporation of isotope was evident 4 to 7 days before transformation was unequivocal by morphologic criteria. The cell count on each infected culture ultimately increased but not until transformation was visually evident and well after the rise in $[^3\text{H}]\text{Tdr}$ incorporation (see Fig. 3). Thus the cell count was not a sensitive indicator of transformation.

Small variations in multiplicity of infection. To determine whether small changes in the quantity of inoculated virus could be detected, the multiplicity of infection was varied in increments from 0.004 to 0.12 $\text{TD}_{50}/\text{cell}$ by varying the amount of virus added to a constant cell concentration. The rate of $[^3\text{H}]\text{Tdr}$ incorporation of each culture was determined 7 days after inoculation and compared with that of a control culture inoculated only with medium. The results (Fig. 4a) showed a linear relationship between the log of the amount of virus inoculated and the log of the $[^3\text{H}]\text{Tdr}$ incorporation measured on day 7. The assay used within these limits can detect at least twofold differences in virus concentration. This result was confirmed in another experiment using fourfold virus dilutions and the A method of

culture (Fig. 4b). From Fig. 4 it can also be seen that, in method A using fewer cells and smaller virus inocula, stimulation of DNA synthesis is not usually detectable until 10 to 15 days after inoculation.

Neutralization of stimulation. To verify that stimulation of $[^3\text{H}]\text{Tdr}$ incorporation was the result of biologically active EBV, a neutralization test was performed. Virus, undiluted and diluted 1:4, was mixed with equal volumes of fourfold dilutions of serum containing EBV antibody or antibody-negative serum or culture medium. The mixtures were incubated for 1 h at 37 C and then inoculated onto leukocyte cultures. The results, shown in Fig. 5, demonstrate that the capacity of EBV, undiluted and diluted 1:4, to stimulate $[^3\text{H}]\text{Tdr}$ uptake was neutralized completely by EBV antibody at 1:4 and 1:16 dilution. Antibody diluted 1:64 reduced stimulation by 60 to 70% whereas the 1:256 serum dilution had no significant effect on $[^3\text{H}]\text{Tdr}$ uptake when compared to cultures inoculated with virus treated with antibody-negative serum or with medium. When undiluted virus was used, neutralization was obvious 7 days after inoculation; when virus was diluted 1:4, DNA stimulation was delayed but neutralization was evident 10 to 15 days after inoculation. Thus virus neutralizing antibodies may be assayed by the inhibition of EBV-induced stimulation of DNA synthesis.

Effects of UV light on stimulation of DNA synthesis by EBV. Cell transformation and stimulation of DNA synthesis by herpes simplex and cytomegaloviruses is effected by UV-treated virus (1, 5); accordingly, we investigated the effects of this treatment on the capacity of EBV to stimulate DNA syn-

FIG. 3. $[^3\text{H}]\text{Tdr}$ incorporation and cell number in infected and uninfected cultures. The data for leukocyte cultures exposed to no virus (●), to undiluted virus (○), and to 10^{-3} diluted virus (Δ) is shown.

FIG. 4. Effect of small variations in multiplicity on the rate of DNA synthesis in infected cultures. (a) Leukocytes separated by Ficoll-Hypaque method and cultured according to method B. [³H]Tdr pulse day 7 is shown. (b) Leukocytes separated by dextran and cultured according to method A. [³H]Tdr pulses day 10 (●), and day 15 (○) after inoculation are shown. Results expressed as net ³H counts/min stimulated; uninfected cell values have been subtracted.

thesis (Fig. 6). Progressively larger doses of UV light applied to virus stocks resulted in decreased ability of the virus to stimulate DNA synthesis. A total of 2,400 ergs (4 min) of irradiation reduced the stimulatory capacity of EBV by more than 10-fold. All activity was eliminated by 16 min of UV light exposure.

Serum requirement for stimulation of [³H]Tdr incorporation. The concentration of fetal calf serum (FCS) in the medium was varied from 20, 10, 5, to 2% (Fig. 7). Stimulation of [³H]Tdr incorporation was greatest in medium containing 20% FCS, whereas in 10 and 5% FCS, it was significantly decreased and in 2% FCS stimulation was completely absent. When the concentration of FCS in infected culture was shifted from 2 to 20% at 24 or 48 h after inoculation, thymidine uptake occurred at a rate slower

FIG. 5. Neutralization of stimulation of DNA synthesis by human serum with EBV antibody: (a) undiluted virus used; (b) virus diluted 1:4. Virus was mixed with medium (●), EBV antibody-negative human serum (▲), or serial fourfold dilutions of antibody-positive human serum 1:4 (□), 1:16 (◁), 1:64 (○), and 1:256 (Δ). The serum virus mixtures were held at 37 C for 1 h before inoculation onto leukocyte cultures according to method A.

FIG. 6. Inactivation of DNA stimulating activity of EBV by UV light. Leukocyte cultures were inoculated with aliquots of EBV exposed to 0, 2, 4, 8, and 16 min, respectively, of UV light at 10 erg/mm² per s. A control culture received only medium. Results are expressed as log₁₀ net ³H-labeled counts per minute (³H-labeled counts per minute of control culture subtracted). Data are for [³H]Tdr pulse 12 days after inoculation. Method A used.

Fig. 7. FCS requirement for immortalization by EBV. Cord leukocyte cultures infected with EBV or not infected were initiated in RPMI-1640 in media with 2, 5, 10, and 20% FCS, respectively. [^3H]Tdr incorporation was measured on day 3, 7, and 10 after inoculation. The results are expressed as net counts per minute for infected cultures (net counts per minute: counts per minute infected culture minus counts per minute uninfected culture). Method B was used for leukocyte cultures. Symbols: Δ , 20% FCS; $\circ$, 10% FCS; $\square$, 5% FCS; $\diamond$, 2% FCS.

than that of infected culture initiated in 20% FCS (data not shown). This effect was most notable 5 days after inoculation. This phenomenon suggests that viral adsorption and possibly penetration occur in medium containing only 2% FCS but that the stimulation of [^3H]Tdr incorporation and cellular proliferation is dependent on the serum concentration, and thus on the physiologic state of the cells.

Estimate of the number of transformed cells. Since [^3H]Tdr incorporation is a measure of the DNA synthetic activity of a mass culture, it was of interest to determine how many cells of such a culture were actually transformed upon exposure to an EBV preparation of known virus content. A standard curve may be constructed relating known numbers of transformed cells to [^3H]Tdr incorporation (a representative experiment is shown in Fig. 8). Aliquots of 0.5 ml containing different numbers of recently transformed cord leukocytes in early log phase growth were mixed with 6×10^6 uninfected cord cells which had been cultivated for 24 h. [^3H]Tdr incorporation was assayed for each cell dilution and also for uninfected cord leukocytes alone. The uninfected cord cells served to simulate conditions in which transformation was measured. Using this curve, it was possible to

estimate the number of transformed cells present in the culture 3 days after inoculation, the earliest time that stimulation was detectable by [^3H]Tdr incorporation (Table 1). Assuming that the number of transformants calculated in this fashion represents, predominantly, initial transformants and not daughter cells, an estimate can be made of the proportion of the initial cell population which was transformed. These calculations suggest that approximately 1% of the cell population was transformed at a multiplicity of 0.05 transforming units/cell; this frequency increased to 3% of the population when the multiplicity was increased threefold.

Another approach to the measurement of transformation efficiency was based on the plating efficiency of limiting dilutions of virus-exposed cells. EBV was added to dextran sedimented cells at a multiplicity of 0.025 transforming units per cell and after 24 h of incubation serial dilutions of the cell suspension were made. Aliquots of each cell dilution were placed on feeder layers of human placental fibroblasts, conditions previously established for cloning lymphoblastoid cell lines at high efficiency (17). The cultures were held for 8 weeks for outgrowth of transformed colonies. The results of two such experiments, presented in Table 2, indicated that even at these low multiplicities of infection approximately 1/200 to 1/500 cells transformed.

DISCUSSION

Measurement of the rate of [^3H]Tdr incorporation in inoculated cultures, compared

Fig. 8. Standard curve relating number of transformed cells to net ^3H -labeled counts per minute (^3H -labeled counts per minute of uninfected cord cells [261] subtracted). [^3H]Tdr incorporation according to method B. One of four experiments is shown.

TABLE 1. Estimate of the number of cells transformed after exposure to EBV based on extrapolation from a standard curve relating net [^3H]Tdr incorporation to known numbers of transformed cord cells

Expt no.	Multiplicity (TD ₅₀ /cell)	Net counts/min (day 3) ^a	Calculated no. of transformants ^b /ml	Starting cell no.	Estimate of fraction of initial population which was transformed
1	0.05	276	1.5×10^4	1.2×10^6	0.013
2	0.06	178	1.0×10^4	1.0×10^6	0.010
3	0.16	800	4.5×10^4	1.2×10^6	0.038
4	0.16	519	3.0×10^4	1.2×10^6	0.025

^a Background of uninfected cord blood leukocytes subtracted: all experiments done with method B.^b From data in Fig. 9.TABLE 2. Estimate of number of cells transformed after exposure to EBV based on outgrowth of colonies under cloning conditions^a

Cells planted per culture	Cultures showing transformation/ cultures planted
Expt 1	
1.5×10^6	2/2
1.5×10^5	4/4
1.5×10^4	4/4
1.5×10^3	5/5
1.5×10^2	2/6
1.5×10^1	1/6
1.5×10^0	0/6
Fraction transformed ^b	0.005
Expt 2	
2×10^5	3/3
2×10^4	3/3
2×10^3	5/5
2×10^2	1/6
2×10^1	0/6
2×10^0	0/6
Fraction transformed ^b	0.002

^a Leukocyte cultures separated by the dextran method were exposed to EBV at a multiplicity of 0.03 TD₅₀ (Expt 1) and 0.12 TD₅₀ (Expt 2). After 24 h the cells were counted and plated on replicate cultures of human placental fibroblasts at the indicated cell dilutions. Cultures were observed for 8 weeks for outgrowth of transformed cultures.

^b Estimate of fraction of initial population transformed. Reed Muench formula was used.

with the rate of isotope incorporation in uninfected cultures, provides a rapid, objective, sensitive, and reproducible assay for permanent lymphocyte proliferation induced by transforming strains of EBV. To assay preparations of unknown virus titer comparison must be made with virus stocks of known DNA stimulation activity and transforming titer. That this reaction is due to biologically active EBV is indicated by several lines of evidence. Extracellular fluid from the nonproducer Raji cell line con-

taining no virions, and extracellular fluid from the P₃J-F-R-1 cell line containing virions which induce only abortive replication and not transformation, do not cause stimulation of DNA synthesis (16). When the inoculum of B95-8 virus is decreased by neutralization with antibody, or by UV-irradiation, the rate of DNA synthesis is decreased in parallel. The inactivation of the transforming properties of B95-8 EBV by UV light contrasts with transformation by ordinarily "lytic" herpes simplex and cytomegalovirus. Genome damage is required before these other herpes viruses can transform hamster cells (1, 5).

Increased rates of incorporation of [^3H]Tdr by EBV-exposed leukocytes reflects, for the most part, cellular DNA synthesis. Gerber demonstrated that the ^3H label found in leukocyte DNA after exposure to EBV had a density in CsCl isopycnic gradients comparable to Simian virus 40 (SV40) form I DNA (6). This result indicated that the composition of the newly made DNA was equivalent to approximately 42 mol% guanine and cytosine (4). EBV DNA contains approximately 59 mol% guanine plus cytosine (21).

Human cord blood leukocytes, transformed by EBV, are nonpermissive hosts, that is they produce neither mature virus nor capsid antigens, although they contain complement fixing antigens including the EB nuclear antigen (14). However, cell lines obtained from cord blood after exposure to the B95-8 strain of EBV contain approximately 30 to 60 "genome equivalents" of EBV DNA per cell on the basis of hybridization between EBV complementary RNA and cellular DNA (Pagano and Miller, unpublished data). The result is obtained after infection at low multiplicity and suggests that amplification of viral DNA occurs during initial steps in transformation. Further work is needed to define precisely the nature of the newly made DNA. New assays will be needed to detect the very small amounts of viral-specific DNA syn-

thesis which may be occurring. Even in cell lines which are productive of mature EBV, viral DNA cannot be detected by the assay of isopycnic centrifugation of total radiolabeled DNA unless cellular DNA synthesis is first inhibited (9). It will also be of interest to measure the relative contributions of semiconservative, repair, and mitochondrial DNA synthesis to the cellular components.

Efficiency of transformation by EBV. A unique feature of the interaction of EBV with human cord blood lymphocytes is the high efficiency of transformation and stimulation of DNA synthesis. With EBV transformation efficiencies of approximately 10^{-2} are achieved with very low multiplicities of infection, never exceeding 10 viral particles/cell. DNA synthesis is stimulated at low multiplicities even by very highly diluted preparations in which the multiplicity may be as low as one particle per 10^4 cells.

In estimating the efficiency of transformation by EBV, the two methods used (Table 1 and 2) provided comparable results. The outgrowth of transformants under cloning conditions results in an underestimation of the number of transformants, since the plating efficiency of freshly transformed cord cells, although not known, is certainly less than 100%. The use of net [^3H]Tdr incorporation by infected cultures 3 days after inoculation to estimate the number of transformed cells may result in an overestimation since some proliferation of the initial transformants may have occurred already. Both types of estimates of efficiency may be affected by the use of a heterogeneous population of leukocytes, some of which may be totally insensitive to EBV.

By contrast to EBV, transformation by papova viruses is inefficient. For example, at multiplicities of 1 PFU/cell (100 particles/cell), the frequency of transformation of BHK-21 cells by polyoma virus is 10^{-4} (12), and at multiplicities of 1,000 PFU/cell (10^5 particles/cell), the efficiency of transformation of mouse 3T3 cells by SV40 (a very sensitive system) is 10^{-2} (22). If human diploid cells are exposed to similar multiplicities of SV40, transformation is about 300 times less efficient than in 3T3 cells (23). The induction of cellular DNA synthesis in mouse kidney cells by polyoma virus requires multiplicities ranging from 0.5 to 1,000 PFU/cell (2).

This comparison of the efficiency of transformation by EBV and the papova viruses may not be entirely valid since the fraction of the viral particle population which is responsible

for transformation is not known for either EBV or the papovavirus system. Nonetheless, the high efficiency of EBV-induced transformation, even at low multiplicities, raises the possibility that there is a fundamental difference in the nature of the mechanism of transformation by the two virus groups. Cells "transformed" by EBV are those which are caused to continue replication in vitro; hence, we have in the past used the term immortalization for this process. Cells transformed by papova viruses acquire new growth properties but are often already capable of continued in vitro growth before exposure to the transforming agent.

Since lymphocytes are the hosts for EBV, one should distinguish between transitory stimulation of DNA synthesis which occurs as part of blastogenesis effected by nonspecific mitogens and specific antigens, and permanent immortalization induced by EBV. The stimulation of DNA synthesis in cells from cord blood by EBV is due to intracellular action of the virus and is distinct from antigenic stimulation which occurs when inactivated EBV is added to cells from immune subjects (7). Comparison of the details of the DNA stimulatory mechanisms of these two types of phenomena might provide further insight into control of lymphocyte proliferation.

ACKNOWLEDGMENTS

We are grateful to J. F. Enders, D. M. Horstmann, J. S. Pagano, and W. C. Summers for critical readings of the manuscript and to L. Heston, C. Hoesli, M. Newmuis, and V. Thompson for cheerful and excellent assistance.

This work was supported by Public Health Service grants CA-12055 and CA-16038 from the National Cancer Institute, GM-20124 from the National Institute of General Medical Sciences, HD-00177 from the National Institute of Child Health and Human Development, and AI-11611 from the National Institute of Allergy and Infectious Diseases; and by American Cancer Society grant VC-107; and Damon Runyon Memorial Fund DRG-1147. G. M. is an Investigator of the Howard Hughes Medical Institute.

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