*Proc. Natl. Acad. Sci. USA*
Vol. 73, No. 9, pp. 3211–3215, September 1976
Cell Biology

# Identification of an intracellular precursor to DNA excreted by human lymphocytes

(phytohemagglutinin/gene amplification)

JOHN C. ROGERS

Divisions of Hematology and Oncology, Washington University School of Medicine, St. Louis, Missouri 63110

*Communicated by Paul A. Marks, June 22, 1976*

ABSTRACT **Phytohemagglutinin-stimulated human peripheral blood lymphocytes *in vitro* synthesize DNA that is excreted into the culture medium. When such cells are pulse-labeled with [$^3$H]thymidine during the peak of DNA synthesis on day 3 of culture, then cultured for 3 more days in the absence of isotope, labeled DNA moves slowly into the Hirt supernatant cell fraction from the pellet fraction containing chromosomal DNA, and then into the culture medium. The number of copies of excreted DNA sequences in the Hirt pellet fraction was determined for lymphocytes harvested on days 3, 4, and 6 after stimulation and compared to the number found in resting lymphocyte DNA and in placenta DNA. While resting lymphocyte and placenta DNAs contain one to two copies of sequences similar to excreted DNA per haploid genome, stimulated lymphocytes on days 3 and 4 of culture contain 3- to 4-fold more copies; by day 6 of culture, stimulated lymphocytes contain only 1- to 2-fold more copies than resting lymphocytes. Thus, phytohemagglutinin induces lymphocytes to selectively replicate several copies of a limited portion of their genome, copies which are then excreted into the culture medium. As determined by reassociation kinetics analysis, a high-molecular-weight DNA fraction from the Hirt supernatant contains sequences found in excreted DNA. This DNA may represent an intermediate formed prior to release of excreted sequences from the cells.**

When human peripheral blood lymphocytes are stimulated *in vitro* by mitogens, part of the DNA that is synthesized is excreted into the culture medium (1). This DNA contains unique sequence elements, which represent about 10% of the lymphocyte genome, and excreted DNA preparations from cultures derived from different lymphocyte donors largely share the same sequences (2). These data suggest that excreted DNA is the product of an unusual process occurring during lymphocyte activation which is different from genome replication for mitosis. This concept so differs from traditional views of lymphocyte biology that rigorous exclusion of artifactual DNA "excretion" has been necessary. While previous control experiments argued against the phenomenon resulting from cell death, some skepticism about its physiological significance remains. Therefore, the current experiments were designed to identify an intracellular precursor of excreted DNA to lend further support to the hypothesis that excreted DNA is important in lymphocyte activation. I have now demonstrated that excreted sequences initially are amplified, since a number of copies of excreted sequences per cell are newly synthesized after lymphocyte stimulation and these sequences can be isolated with the bulk chromosomal DNA. In addition, the experiments identify an intracellular form of excreted sequences separable from bulk chromosomal DNA that may be an intermediate precursor to the DNA released into the culture medium.

Abbreviations: PHA, phytohemagglutinin; dT, thymidine; SSC, 0.15 M NaCl–0.015 M Na citrate; $NaDodSO_4$, sodium dodecyl sulfate; $C_0t$, initial concentration of DNA (mol of nucleotide/liter) × sec.

## MATERIALS AND METHODS

**Lymphocyte Cultures.** Human peripheral blood lymphocytes were purified and cultured as described (2). Cultures were stimulated on day zero by adding to each tube 2.5 μg of the leukoagglutinating-phytohemagglutin (L-PHA) of *Phaseolus vulgaris* purified from PHA-P (Difco) by the method of Weber *et al.* (3); this quantity caused about half-maximal uptake of [$^3$H]thymidine (dT) on day 3. Cultures were derived from three different lymphocyte donors, A, B, and C, and are identified by these letters. Cultures A and B were grown initially without isotope, but were then pulse-labeled for 4 hr on day 3 with 3 μCi of [*methyl*-$^3$H]dT, 6.7 Ci/mmol (New England Nuclear) (added in 0.1 ml of sterile 0.9% NaCl). Four volumes of sterile 0.9% NaCl were then added to each tube; the cells were harvested by centrifugation at 300 × *g* for 10 min and were resuspended in fresh medium without isotope. In other cultures [$^{14}$C]dT (culture C) or [$^3$H]dT (culture C-2) was present throughout as before (2).

**Purification of DNA.** DNA was purified from culture medium (called *medium* DNA) by treatment with Pronase, extraction with chloroform–isoamyl alcohol, and precipitation as before (2). Then the preparation was dissolved in SSC (0.15 M NaCl–0.015 M Na citrate), mixed CsCl and ethidium bromide to achieve a final density of 1.55 g/ml and 340 μg/ml of ethidium bromide, and centrifuged for 24 hr at 38,000 rpm, 20°, in a Beckman SW 50.1 rotor in polyallomer tubes that had been boiled for 30 min in 0.01 M EDTA, pH 7.0. The tubes were transilluminated with long wave UV light (C-50 transilluminator, Ultra-Violet Products, San Gabriel, Calif.), and the region of the gradient containing the visible fluorescent DNA was removed with a pasteur pipette. Ethidium bromide was removed by extracting the solution twice with equal volumes of *n*-butanol. The DNA solution was dialyzed against 1/10 SSC–0.25% $NaDodSO_4$, then further purified on hydroxyapatite as described (2).

DNA was precipitated by adding 1/10 volume of 2 M NaOAc, pH 6.0, and 2.5 volumes of ethanol; precipitates were dissolved in 1 mM EDTA, pH 7.0, and stored at 4° over a drop of chloroform. DNA purity was monitored as described (2).

**Fractionation of Cellular DNA.** Cellular DNA was routinely separated into two fractions by the method of Hirt (4). Cells pooled from variable numbers of culture tubes were washed (2) and suspended in 0.9% NaCl at a concentration of about 0.2 × $10^8$ cells per ml. To 2.5 ml of the cell suspension in a 50-ml polycarbonate centrifuge tube was added 22.5 ml of 0.01 M Tris·HCl, pH 7.5–0.01 M EDTA – 0.6% $NaDodSO_4$. The suspension was mixed gently by inverting the tube, then was allowed to sit 15 min at room temperature. Six milliliters of 5 M NaCl were then added. The suspension was mixed gently, stored at 4° for 16 hr, and then centrifuged at 4°, 12,000 × *g*,

for 20 min. The supernatant was poured off and the pellet was washed by adding 5 ml of 0.01 M Tris·HCl, pH 7.5–0.01 M EDTA; after centrifuging again, the supernatants were pooled.

DNA in the pellet, called *cell pellet* DNA, was purified as described for whole cell DNA (2).

DNA in the supernatant, called cell *supernatant DNA*, was treated with Pronase, extracted with chloroform-isoamyl alcohol, precipitated, dissolved, and treated with RNase (2); the solution was then made 0.5% with respect to $NaDodSO_4$ and 1 M with respect to $NaClO_4$, extracted twice with chloroform–isoamyl alcohol, dialyzed against SSC, and then concentrated by dialysis against powdered polyethylene glycol (Carbowax 6000, Union Carbide). Aliquots of the concentrated solution were centrifuged on CsCl–ethidium bromide as above; the DNA was then dialyzed against $\frac{1}{10}$ SSC–0.25% $NaDodSO_4$ and precipitated.

Some preparations of supernatant DNA were further fractionated by electrophoresis on 0.4 cm thick, 13 cm wide, 10 cm long agarose (Seakem, Marine Colloids Inc., Rockland, Me.) slab gels as described (5). Fractions were isolated by cutting out appropriate fragments of gels and homogenizing the fragments in a siliconized glass Dounce homogenizer in an equal volume of 5 M $NaClO_4$ at 25°. The homogenate was exhaustively dialyzed against SSC–0.25% $NaDodSO_4$; agarose particles were removed by centrifuging the suspension at 12,000 × *g* for 20 min. The clear supernatant was concentrated, dialyzed against SSC, then centrifuged in CsCl–ethidium bromide. DNA was isolated from gradients as described above. Recovery of DNA initially applied to gels was estimated to be 50%.

Whole cell DNA was isolated from uncultured resting human peripheral blood lymphocytes (2), and placenta DNA was purified from fresh human placentas obtained from the Obstetrics Division of Barnes Hospital.

**Reassociation of DNA.** Methods of analysis of reassociation kinetics have been described (2). Whole cell DNA, and cell pellet and supernatant DNA were sheared to 7S size by sonication (2) prior to use in reassociation experiments.

## RESULTS

Earlier work (2) suggested that PHA-stimulated lymphocytes selectively excrete a certain portion of their genome. If that interpretation is true, an intracellular precursor must exist which, since it must be transported to the plasma membrane, should be separable from chromosomal DNA. The method of Hirt (4) permits separation of relatively small DNA molecules that are not covalently attached to the very large chromosomal DNA molecules. Accordingly, this method was used in an attempt to identify such a precursor to excreted DNA. Previous work demonstrated that stimulated lymphocytes pulse-labeled on day 3 of culture excreted most of the labeled DNA during the remainder of the culture period (1). Therefore, experiments were performed (see Fig. 1) that analyzed the fate of DNA in cells pulse-labeled with [$^3$H]dT for 4 hr on day 3 of culture; $\frac{1}{4}$ of the cells were harvested immediately after being washed and resuspended in isotope-free medium and the remaining cells were harvested on days 4, 5, and 6. The total amount of acid-precipitable [$^3$H]dT in the medium and the cell pellet and supernatant was determined each day. The total quantity of DNA in each fraction was calculated using the specific activity of the respective purified DNAs. The total amount of DNA in the cell pellet remained relatively constant from day 3 to day 6 (Fig. 1A), but the amount of label in that fraction declined progressively such that on day 6 only about 25% of the initial quantity remained (Fig. 1B). In contrast, the amount of DNA in the

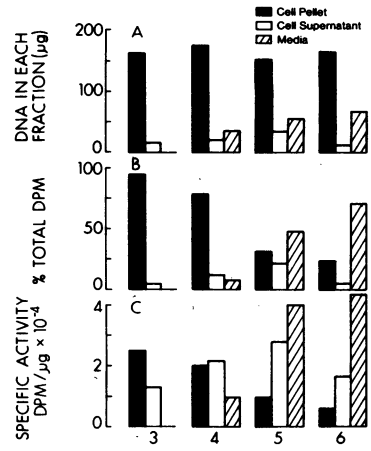


FIG. 1. Quantitation of [$^3$H]dT and of DNA in culture media and cell fractions. Donor A lymphocytes, 1.5 × $10^6$ in 2 ml of medium per culture tube, were stimulated with L-PHA on day zero, pulse-labeled for 4 hr on day 3 with [$^3$H]dT, washed, and resuspended in fresh medium without isotope (see *Materials and Methods*). Twenty-three culture tubes were harvested immediately after the cells were resuspended on day 3, and at 24-hr increments thereafter. The quantities of DNA and of [$^3$H]dT in DNA in the culture medium and in the cell pellet and cell supernatant fractions were determined (see *Materials and Methods*). Total dpm recovered were: day 3, 1.71 × $10^6$; day 4, 1.74 × $10^6$; day 5, 1.87 × $10^6$; and day 6, 1.68 × $10^6$.

culture medium increased progressively from none on day 3 to an amount representing 28% of the total quantity of DNA in the culture on day 6 (Fig. 1A). The quantity of [$^3$H]dT in culture medium DNA similarly increased from none on day 3 to an amount representing 70% of the total present in the culture on day 6 (Fig. 1B). The specific activity of DNA isolated from the medium on day 6 was twice that of the cellular DNA isolated immediately after the cells were pulse-labeled on day 3 (Fig. 1C). Interestingly, the quantity of DNA, the quantity of [$^3$H]dT in DNA, and the specific activity of DNA in the cell supernatant fraction increased from day 3 to day 5, then decreased on day 6. Culture B gave similar results (data not presented). These data were interpreted to mean that most of the DNA being synthesized on day 3 was destined to be excreted, and that, while this DNA initially was associated with the cell pellet fraction, it appeared to be released slowly, first into the cell supernatant fraction and then into the medium. Release of DNA from the cell pellet fraction must be nonrandom, otherwise unlabeled DNA would have rapidly diluted out the labeled sequences in these fractions.

To document that the DNA sequences being synthesized on day 3, the DNA sequences appearing in the cell supernatant fraction, and the DNA sequences appearing in the culture medium were in fact similar, I first quantitated the number of copies of sequences similar to excreted DNA present in cell pellet DNA at various times after stimulation, and then compared cell supernatant DNA to excreted DNA to determine if they shared the same sequences.

Lymphocytes from donor C, cultured in the constant presence of [$^{14}$C]dT, were harvested on day 6 and DNA was purified from the cell pellet, cell supernatant and medium. The reassociation pattern for this medium DNA (Fig. 2A) contains a major component representing 49% of the DNA with a $C_0t_{1/2}$ of 99 mol·sec/liter (Fig. 2A and Table 1). $C_0t$ is the initial concentration of DNA (mol of nucleotide/liter) × sec.

A second culture was obtained from donor C and cultured in the constant presence of [$^3$H]dT. Medium DNA from this culture, called C-2 medium DNA, reassociated with a major

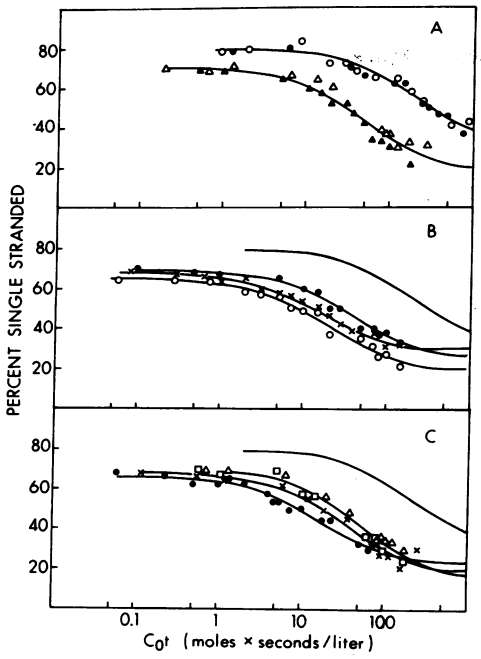


FIG. 2. Reassociation analysis of C medium DNA. Medium DNA was purified on day 6 from donor C lymphocytes cultured in the constant presence of [$^{14}$C]dT. Reassociation analysis was performed as described (2). Data are plotted as the percent of single-stranded DNA against $C_0t$ (6); solutions are derived from a modification (2) of Britten's "Finger" computer program (7) and represent the best least squares fit to the data. The solution for each experiment includes the corrected $C_0t_{1/2}$ value (2), the standard error for that value (2), and the root mean square (RMS) for the computer data fit. (A) C medium DNA, 8.6 μg, in 15.0 μl (○), or C medium DNA, 8.6 μg, plus 86 μg of salmon sperm DNA, in 15.0 μl (●), reassociated with a $C_0t_{1/2}$ = 98.7 ± 2.8 mol·sec/liter, RMS = 2.49%. C medium DNA, 12.4 μg, plus 96 μg of resting lymphocyte DNA, in 33.4 μl (△) or in 15.0 μl (▲), reassociated with a $C_0t_{1/2}$ = 24.0 ± 0.9 mol·sec/liter, RMS = 3.46%. (B) C medium DNA, 9.6 μg, plus culture A (Fig. 1) day 3 cell pellet DNA, 67.4 μg, in 30.0 μl (○), reassociated with a $C_0t_{1/2}$ = 9.8 ± 0.3 mol·sec/liter, RMS = 1.87%. C medium DNA, 9.6 μg, plus culture A day 4 cell pellet DNA, 84.6 μg, in 30.0 μl (×), reassociated with a $C_0t_{1/2}$ = 6.6 ± 0.2 mol·sec/liter, RMS = 1.60%. C medium DNA, 9.6 μg, plus culture A day 6 cell pellet DNA, 72.3 μg, in 30 μl (●), reassociated with a $C_0t_{1/2}$ = 16.4 ± 0.4 mol·sec/liter, RMS = 1.48%. (C) C medium DNA, 9.6 μg, plus culture B day 4 cell pellet DNA, 95.8 μg, in 34.3 μl (●), reassociated with a $C_0t_{1/2}$ = 6.5 ± 0.2 mol·sec/liter RMS = 2.22%. C medium DNA, 12.4 μg, plus culture B day 6 cell pellet DNA, 65.5 μg, in 37.8 μl (×), reassociated with a $C_0t_{1/2}$ = 16.9 ± 0.9 mol·sec/liter, RMS = 2.45%. C medium DNA, 12.4 μg, plus placenta DNA, 96.8 μg, in 31.7 μl (△) or in 15.0 μl (□), reassociated with a $C_0t_{1/2}$ = 27.7 ± 0.8 mol·sec/liter, RMS = 2.33%.

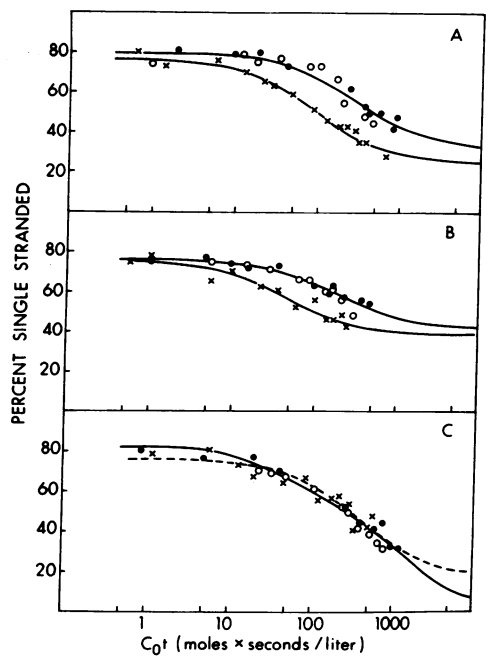


FIG. 3. Reassociation analysis of C-2 medium DNA and C cell supernatant DNAs. Data plotting and analysis are described in Fig. 2. (A) C-2 medium DNA, 6.2 μg, plus salmon sperm DNA, 62 μg, in 15.0 μl (○), or C-2 medium DNA, 12.3 μg, in 15.0 μl (●), reassociated with a $C_0t_{1/2}$ = 133 ± 4.2 mol·sec/liter, RMS = 2.61%. C medium DNA, the marker DNA, 8.6 μg, plus 16.8 μg of C-2 medium DNA, in 19.7 μl (×), reassociated with a $C_0t_{1/2}$ = 49 ± 1.6 mol·sec/liter, RMS = 2.15%. (B) The high-molecular-weight fraction of C cell supernatant DNA, 4.9 μg, in 15.0 μl (●), or 4.9 μg plus 49 μg of salmon sperm DNA, in 15.0 μl (○), reassociated with a $C_0t_{1/2}$ = 63 ± 1.3 mol·sec/liter, RMS = 1.48%. The high-molecular-weight cell supernatant DNA, 4.9 μg, plus C-2 medium DNA, 9.8 μg, in 15.0 μl (×), reassociated with a $C_0t_{1/2}$ = 15.8 ± 0.6 mol·sec/liter, RMS = 2.10%. (C) The low-molecular-weight fraction of C cell supernatant DNA, 8.5 μg, plus salmon sperm DNA, 85 μg, in 15.0 μl (○), or low-molecular-weight cell supernatant DNA, 12.7 μg, in 15.0 μl (●), or low-molecular-weight cell supernatant DNA, 8.5 μg, plus C-2 medium DNA, 16.8 μg, in 18.3 μl (×), reassociated in a pattern that could be fit almost equally well by two solutions: a single component with a $C_0t_{1/2}$ = 216 ± 14 mol·sec/liter, RMS = 6.95% (dashed line) or two components, the first containing 22% of the DNA with a $C_0t_{1/2}$ = 9.6 mol·sec/liter and the second containing 61% of the DNA with a $C_0t_{1/2}$ = 698 mol·sec/liter, RMS = 7.00% (solid line). Addition of C-2 medium DNA (×) did not significantly alter this pattern; it has therefore been plotted with the other data.

component representing 47% of the DNA and with a $C_0t_{1/2}$ of 133 mol·sec/liter (Fig. 3A). The two $C_0t_{1/2}$ values, although similar, should be identical if sequences in the two major components are the same. Each curve is defined by the results of two separate experiments performed with and without a 10-fold excess of sheared salmon sperm DNA (2) to control for increased viscosity in subsequent experiments. The low root mean square and standard error values for each solution confirm that data scatter alone cannot account for the two different $C_0t_{1/2}$ values. In addition, the presence of C-2 medium DNA accelerated the reassociation of the major component of C medium DNA (Fig. 3A) by a factor approximately that which would be expected (2) if the two preparations largely shared the same sequences.

The discrepancy in $C_0t_{1/2}$ for C and C-2 medium DNAs can be better understood by considering the complexity of this system. DNA sequences in the major component have been studied because they are identifiable by reassociation analysis. These are a population of sequences that appear homogeneous because they fit the curve for an ideal second-order reaction. However, the sensitivity of analysis would not identify subpopulations with slightly different reassociation rate constants. The $C_0t_{1/2}$ value for a major component therefore represents an average for the entire population. In addition, other populations of sequences are present in medium DNA, reassociate within the $C_0t$ ranges studied, and overlap with those in the major component. First, about 20% of medium DNA reassociates by a $C_0t$ of 1 mol·sec/liter (Figs. 2A and 3A); these presumably represent repetitive sequences. When reassociation of medium DNA is driven by excess cellular DNA in the following experiments, this figure changes, and about 30% of the medium DNA reassociates by a $C_0t$ of 1 mol·sec/liter (Fig. 2A–C). This indicates that repetitive sequences contribute to the initial part of the major component curve. Second, a variable fraction of DNA in medium DNA preparations remains unreassociated within the $C_0t$ ranges studied here. Previous work

Table 1. Estimation of the number of copies of excreted DNA sequences in various DNA preparations

| Marker DNA | Test DNA | Fraction in major component | Measured $C_0t_{1/2}$ | Expected $C_0t_{1/2}$ | Ratio: Expected/ measured |
|---|---|---|---|---|---|
| Culture C medium DNA | None or salmon sperm DNA | 0.49 | 99 | — | — |
| | Unstimulated lymphocyte DNA | 0.53 | 24 | 41 | 1.7 |
| | Placenta DNA | 0.56 | 28 | 44 | 1.6 |
| | Culture A cell pellet DNA | | | | |
| | Day 3 | 0.46 | 9.8 | 44 | 4.5 |
| | Day 4 | 0.39 | 6.6 | 38 | 5.8 |
| | Day 6 | 0.44 | 16 | 42 | 2.6 |
| | Culture B cell pellet DNA | | | | |
| | Day 4 | 0.43 | 6.5 | 35 | 5.5 |
| | Day 6 | 0.50 | 17 | 51 | 3.0 |

The number of copies of sequences similar to excreted DNA were calculated as described (2) from the formula: expected acceleration factor = [(μg test DNA × fraction in major component)/(μg marker DNA × fraction in major component)] × [($C_0t_{1/2}$ marker major component)/($C_0t_{1/2}$ test major component)] + 1. Data for calculations were derived from the experiments illustrated in Fig. 2 assuming that the unique sequence component of cellular DNA represents a fraction of 0.68 and has a $C_0t_{1/2}$ of 770 mol·sec/liter (2). The expected $C_0t_{1/2}$ value is obtained by dividing the original marker DNA $C_0t_{1/2}$ by this factor and represents that which would be obtained if the marker DNA sequences were present in only one copy per haploid genome of test DNA.

(2) demonstrated that this fraction contains cellular DNA sequences of high complexity. These sequences may reassociate within a $C_0t$ range that overlaps with the terminal portion of the major component curve, particularly when reassociation of the major component is driven by excess cellular DNA (2). It has not been possible to achieve $C_0t$ values much greater than 1000 mol·sec/liter since only relatively small amounts of medium DNA have been available. For that reason, the complex transition between the major component and sequences of higher complexity has not been measured accurately. Determination of a corrected $C_0t_{1/2}$ value for the major component depends upon accurately estimating the fraction of DNA within that component. These overlapping populations impose an error on that estimate which can be minimized by computer analyses of the curves (2), but such analyses provide only a reasonable approximation of the true values since they may oversimplify a more heterogeneous system.

In the following experiments (Fig. 2A–C) the reassociation of C medium DNA was driven by excess cellular DNA from different sources to determine the number of copies of excreted sequences present in these cellular DNAs. A model formula (2) was used to predict the $C_0t_{1/2}$ value that should be obtained for the major component in each experiment if each cellular DNA sample contained one copy per haploid genome. A lower $C_0t_{1/2}$ value than expected indicates that more copies may be present; these results are tabulated in Table 1. It should be recognized that these calculations are strictly true only for a homogeneous probe, such as a viral DNA (8), and that, in this complex system, the figures represent approximations. However, the experiments are valid since one probe, C medium DNA, was used throughout, the only variable being the cellular DNA added to it. The important conclusions are derived by comparing results of experiments using cell pellet DNA from stimulated lymphocytes to results using resting lymphocyte or placenta DNAs.

An excess of sheared cellular DNA from unstimulated lymphocytes drove the reassociation of C medium DNA by a factor 1.7 times that which theoretically would be obtained if only one copy of the excreted sequences were present per haploid resting lymphocyte genome (Fig. 2A and Table 1) (2). C medium DNA was allowed to reassociate in the presence of sheared cell pellet DNA isolated on days 3, 4, and 6 from culture A (Fig. 1). These results (Fig. 2B and Table 1) demonstrate that day 3, day 4, and day 6 cell pellet DNAs accelerated the reassociation of the major component of C medium DNA by respective factors of 4.5, 5.8, and 2.6 times that expected if excreted sequences were present only once per haploid cell genome. Similarly, sheared cell pellet DNA isolated on day 4 and day 6 from culture B, a culture prepared and harvested in a manner identical to culture A, accelerated the reassociation of C medium DNA (Fig. 2C and Table 1) by respective factors of 5.5 and 3.0 times that expected. Human placenta DNA (Fig. 2C and Table 1) accelerated the reassociation of C medium DNA by a factor 1.6 times the rate for a single copy per haploid genome. Thus, the stimulated lymphocytes on days 3 and 4 contained, in the cell pellet fraction, 2.7 to 3.5 times more copies of excreted DNA sequences per haploid genome than did resting lymphocytes or placenta cells; by day 6, the number of copies had decreased to 1.6 to 1.8 times per haploid genome. These findings are compatible with pulse-label data from culture A.

Next, cell supernatant DNA was analyzed. On agarose gel electrophoresis, a variable portion formed a high-molecular-weight band near the top of the gel, at the resolution limit, while the remainder was distributed over the length of the gel up to the bromphenol blue marker (data not shown). Physical characterization and subcellular localization of this DNA will be reported elsewhere. In contrast, cell pellet DNA did not enter a similar gel, while medium DNA accompanied the bromphenol blue marker (data not shown). Cell supernatant DNA (216 μg) from culture C was fractionated by electrophoresis on a 1% agarose gel into a high-molecular-weight fraction containing the band in the second 1 cm from the top and a lower-molecular-weight fraction containing the rest of the DNA on the gel encompassing 5 cm from the first fraction to the bromphenol blue marker. The latter fraction presumably would contain any nonspecifically adsorbed medium DNA. Recovery

of purified DNA was, for the high-molecular-weight fraction, 19.7 μg, and for the low-molecular-weight fraction, 85.6 μg. The DNA recovered from each fraction was sheared by sonication and studied by reassociation analysis to determine which contained sequences similar to excreted DNA.

The high-molecular-weight fraction of C cell supernatant DNA contained a major component representing 34% of the DNA that reassociated with a $C_0t_{1/2}$ of 63 mol·sec/liter (Fig. 3B). In the presence of C-2 medium DNA, the reassociation of this component was significantly accelerated (37% of the DNA was present in the major component with a $C_0t_{1/2}$ of 16 mol·sec/liter), indicating that the two preparations largely share similar sequences (Fig. 3B). The lower-molecular-weight fraction of C cell supernatant DNA reassociated in a pattern that could be fit equally well by a solution predicting one component of 59% having a $C_0t_{1/2}$ of 236 mol·sec/liter with 17% remaining unreassociated, or two components, one of 22% with a $C_0t_{1/2}$ of 9.6 mol·sec/liter and one of 61% with a $C_0t_{1/2}$ of 698 mol·sec/liter with none remaining unreassociated (Fig. 3C). C-2 medium DNA did not significantly alter this pattern (Fig. 3C), demonstrating that the two preparations shared few sequences.

## DISCUSSION

The reassociation analysis experiments are open to quantitative uncertainties as discussed above. These uncertainties are demonstrated by the results obtained when the reassociation of C medium DNA was driven by resting lymphocyte DNA and by placenta DNA; the ratios, expected $C_0t_{1/2}$/measured $C_0t_{1/2}$, were 1.7 and 1.6, respectively, but theoretically should have been 1.0 (2). Either resting lymphocytes and placenta cells may carry more than one copy of excreted sequences per haploid genome or, more likely, these results reflect a systematic error imposed by the complex system under study. However, compared to the results obtained with resting lymphocyte DNA, stimulated lymphocyte cell pellet DNA contained 3- to 4-fold more copies of excreted DNA on days 3 and 4 of culture; by day 6 this declined to a 1- to 2-fold difference. As the number of excreted DNA sequences declined in the cell pellet fraction, they could be identified in the cell supernatant fraction and accumulated in the culture medium. This is strong evidence that the stimulated lymphocyte may selectively replicate and excrete a limited portion of its genome. Cell death cannot account for selective replication. Presumably excreted sequences identified in the cell supernatant fraction represent an intermediate form en route from the nucleus to the plasma membrane, but no conclusion with regard to the site or mechanism of replication of these sequences can be drawn from these data.

The complexity of sequences replicated and excreted by an individual cell remains unknown. As previously discussed (2), there are two possibilities: either each cell may replicate several copies of about 10% of its genome, or each cell may replicate a large number of copies of a very small part of its genome if different cells replicate different sequences.

There is only limited information from other systems that might help explain this phenomenon. During early meiosis, amphibian oocytes selectively replicate genes for rRNA (9, 10); this is the only model for what has been defined as gene amplification (9). Since at present no function is known for excreted DNA sequences, they cannot be called genes, but they are amplified in an unusual way and differ in this regard from cytoplasmic membrane-associated DNA sequences described in a long-term human lymphoblast cell line (11, 12). This entire process, activation of a resting cell by an external stimulus followed in turn by morphologic alteration, DNA replication, including selective replication of a number of copies of a limited part of the genome, then release of this DNA into the culture medium, has similarities to that of activation and replication of a latent DNA virus (13, 14) but clearly involves unique cellular DNA sequences of high complexity. A better understanding of this phenomenon may be of importance in the general fields of lymphocyte physiology and the immune response.

I am indebted to Mrs. Fu-Mei Lo for expert technical assistance, and to Dr. L. Gelb for helpful discussions. This investigation was supported by NIH Grant CA 17143 and by American Cancer Society JFCF 275A.

1. Rogers, J. C., Boldt, D., Kornfeld, S., Skinner, A., Sr. & Valeri, C. R. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1685–1689.
2. Rogers, J. C. (1976) *J. Exp. Med.* **143**, 1249–1264.
3. Weber, T., Nordman, C. T. & Gräsbeck, R. (1967). *Scand. J. Haematol.* **4**, 77–80.
4. Hirt, B. (1967) *J. Mol. Biol.* **26**, 365–369.
5. Helling, R. B., Goodman, H. M. & Boyer, H. W. (1974) *J. Virol.* **14**, 1235–1244.
6. Britten, R. J. & Kohne, D. E. (1968) *Science* **161**, 529–540.
7. Britten, R. J., Graham, D. E. & Neufeld, B. R. (1974) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 29, pp. 363–418.
8. Gelb, L. D., Kohne, D. E. & Martin, M. A. (1971) *J. Mol. Biol.* **57**, 129–145.
9. Brown, D. D. & Dawid, I. B. (1968) *Science* **160**, 272–280.
10. Gall, J. G. (1968) *Proc. Natl. Acad. Sci. USA* **60**, 553–560.
11. Lerner, R. A., Meinke, W. & Goldstein, D. A. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 1212–1261.
12. Meinke, W. & Goldstein, D. A. (1974) *J. Mol. Biol.* **86**, 757–773.
13. Hampar, B., Derge, J. G., Martos, L. M. & Walker, J. L. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 78–82.
14. Gerber, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 83–85.