Unstable high molecular weight inverted repetitive DNA in human lymphocytes

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ABSTRACT

About 1% of newly synthesized DNA from PHA-stimulated human lymphocytes can be isolated as large (up to 90 kilobase pairs) double stranded fragments that resist sequential alkali and heat denaturation steps but are not closed circular. By electron microscopy about 1% have single-strand hairpin loops at one end and therefore represent inverted repetitive sequences (IR-DNA). Most of the remainder have a blunt-appearing double-strand terminus at both ends (78%) or one end (18%). Indirect evidence indicates that these also are inverted complementary structures with terminal hairpin loops too small to be visualized: (1) Treatment with either a 5' or 3' single-strand exonuclease generates essentially only fragments with a single strand at one end; (2) with partial denaturation, the number of fragments with identifiable single-strand hairpin loops increases (to about 20%); (3) after S₁ nuclease digestion, >95% can be fully heat denatured. C₀t analysis indicates that these fragments are derived from dispersed sites throughout the genome. Up to 25% of DNA released from lymphocytes during growth similarly resists denaturation, and released DNA and IR-DNA are both enriched in the same set of repetitive sequences. Thus at least a portion of IR-DNA appears to be unstable.

INTRODUCTION

We have previously described an unusual phenomenon whereby normal human peripheral blood lymphocytes stimulated with phytohemagglutinin (PHA) (1) to grow in culture synthesize some DNA in excess of the need for mitosis (2) and release it into the culture medium (3,4). As assessed by reassociation (Cot) analysis, the overall sequence complexity of released DNA was limited, representing about 10% of the whole genome (2,3); when the unique sequence component present in released DNA was used as a reassociation probe, these sequences were apparently amplified 3-4 fold during active growth (2,5). Although other studies (5-7) indicated that DNA release was under certain constraints and could not be explained by lysis of dead cells, we had no explanation why lymphocytes should intentionally amplify a relatively enormous amount of their genome.

Results in other, well defined systems involving gene amplification

might explain our observations and make predictions that might be more readily tested in our system. In particular, Kaufman, *et al.* (8) suggested that cells might randomly amplify portions of their genome during growth; therefore cells that by chance amplified the gene for the protective enzyme, dihydrofolate reductase, would survive under gradual imposition of selective pressure with the folic acid analogue, methotrexate (8,9). They speculated that a mechanism similar to that proposed by Botchan, *et al.* (10) for amplification of SV40 might pertain; under this "onionskin" model, repeated rounds of replication occur at an origin of replication before the original newly synthesized strands can fuse with those from an adjacent replicon (10).

We hypothesized that such a mechanism might explain the amplification observed in lymphocytes. One possible consequence of this mechanism might be the formation of physically distinct amplification products. As outlined in Figure 1, theoretically the amplified strands would be in close proximity and might have one or both ends ligated together. The resulting structure would be a large inverted repetitive molecule; in the latter instance it would also be closed circular.

The work presented here demonstrates that very large inverted repetitive DNA molecules can be isolated from growing lymphocytes and represent about 1% of newly synthesized DNA. These structures (termed IR-DNA) contain sequences dispersed throughout the genome and may represent the genome at random. Released DNA is enriched in high molecular weight inverted repetitive molecules, and IR-DNA isolated from nuclei of growing cells is enriched in repetitive sequences shared by released DNA. Therefore, released DNA, at least in part, may be derived from structures that conceptually should be free from the chromosome and therefore unstable.

MATERIALS AND METHODS

Cell Cultures

Peripheral blood lymphocytes from normal human volunteers were isolated and stimulated with PHA in culture as described (5). Some cultures were labeled throughout the culture period with 0.25 $\mu\text{Ci/ml}$ ^3H -thymidine (New England Nuclear, Boston, MA) diluted to 50 mCi/mmol (5).

DNA

Whole cell DNA and trypsin-released DNA were isolated as before (5). Mitochondrial DNA was purified from human placenta (11). Inverted repetitive DNA (IR-DNA) was isolated from day 3 lymphocyte nuclei (12). In general, nuclei from 5×10^7 lymphocytes in a 50 ml plastic conical tube ($\alpha 2070$,

Figure 1 - Model for generation of high molecular weight inverted repetitive sequences: (A) A second round of replication is initiated within a replicon before the strands synthesized during the first round ligate to those in flanking replicons. This is identical to the structure proposed by Botchan, *et al.* (10) to explain excision of SV40 from chromosomes. (B) The strands resulting from the second round of replication are free from the chromosome; theoretically they would be in close proximity and might have their ends ligated together as indicated by the dotted lines. Each strand would then be composed of an inverted repetitive sequence schematically presented where ABC and A'B'C' are complementary and would base pair A to A', etc.

Falcon, Oxnard, CA) were resuspended in 1 ml 0.01 M Tris-HCl pH 7.4 - 0.01 M NaCl - 1.5 mM MgCl₂ - 1% NP 40 - 9% sucrose by vigorous pipetting, then diluted with 12.5 ml 0.01 M Tris pH 7.4 - 0.01 M EDTA - 0.6% sodium dodecyl sulfate. One and a half ml of pronase (Protease Type VI, Sigma, St. Louis, MO), 5 mg/ml stock solution, was added and the tube was incubated 2 hours, 37°C. The DNA was then sheared by vortexing 45 sec at full speed on a Vortex-Genie Mixer (Scientific Industries, Springfield, MA). Twenty ml aliquots of sheared DNA were denatured by adding 0.36 ml 3N NaOH and mixed by

inverting gently. This resulted in a final pH of about 12.5. The tubes were incubated at room temperature for 10 min, then neutralized to pH 8.5-9 by the addition of 0.4 ml 2 M Tris-HCl, pH 7.0 to each. After sitting 3 min, 2.2 ml of 5 M NaCl was added to each and the samples were extracted with an equal volume of phenol previously saturated with 3% NaCl (13). Single stranded DNA is extracted with the phenol in this step (13). The aqueous layer was recovered after centrifugation 10 min at 2600 x g, extracted with an equal volume of phenol:chloroform:isoamyl alcohol 50:50:1, and then extracted again with ether. DNA was precipitated with powdered polyethylene glycol (14), or with 2 volumes of ethanol. After collection by centrifugation, the DNA was suspended in 1 mM EDTA pH 7.4, heat denatured at 100° for 10 min, and then chilled rapidly in an ice-H₂O slurry. Alternatively, some preparations (Fig. 2) were exposed to a second alkali denaturation step, since heating at neutral pH will itself induce nicks (16). Fully double-stranded molecules were then isolated by S₁ nuclease digestion.

Isolation of S₁-Resistant High Molecular Weight Inverted Repetitive DNA

For preparative use, after heat denaturation, inverted repeat enriched DNA preparations were incubated with a 10-fold excess of S₁ nuclease (Miles, Elkhart, IN) in 0.025 M potassium acetate - 0.1 mM ZnSO₄ - 0.15 M NaCl for 30 min at 37°C. The incubation was stopped by the addition of EDTA to 0.05 M and Tris-HCl pH 7.0 to 0.1 M. After extraction with an equal volume of phenol:chloroform:isoamyl alcohol 50:50:1 the S₁-resistant DNA was either applied directly to an A-50M column (see below) or precipitated at -20°C in the presence of 3 volumes of ethanol. After collection by centrifugation the precipitate was taken up in a maximum volume of 0.5 ml 1 mM EDTA pH 7.4 and applied to a 1 cm x 18 cm column of BioGel A50M (BioRad, Richmond, CA) in 0.2 mM EDTA - 5 mM Tris-HCl pH 7.4. After collection of 1 ml fractions, high molecular DNA eluting with the exclusion volume was identified and precipitated with 3 volumes of ethanol at -20°C in the presence of 0.2 M sodium acetate pH 6.0.

Electron Microscopy

Electron microscopy was performed according to the methods of Davis *et al.* with modifications (15). The hyperphase spreading solution was 0.1 M Tris-HCl pH 8.4, 0.01 M EDTA, 40% formamide, 0.5-0.8 µg/ml DNA, and 75 µg/ml cytochrome C. Fifty µl were spread onto a hypophase of distilled water or 10% formamide in distilled water. In some experiments, 0.1 M NaHCO₃ pH 9.3 was substituted for the Tris buffer in the hyperphase to increase contrast between single- and double-stranded DNA. Spread DNA was picked up on freshly

prepared parlodion-coated grids, stained 30 sec in 50 μ M uranyl acetate in 90% ethanol, rinsed briefly in ethanol, dried, rotary shadowed at 8-10° with platinum/palladium, and viewed in a Philips EM 201 electron microscope. Partial denaturation (17) was carried out for 10 minutes at 30° in 7.2 M NaClO₄, 0.01 M Na acetate pH 7.0, 0.002 M EDTA, and 6% formaldehyde. The sample (100 μ l) was then dialyzed for 30 min at 4°C on a VSWP025 filter (Millipore, Bedford, MA) floating on 40% deionized formamide--0.01 M Na acetate pH 7.0.

Exonuclease Digestions

E. coli Exonuclease III and 5'-Exonuclease from bacteriophage λ were from New England Biolabs, Beverly, MA, and units are as defined by that supplier. Digestions were carried out in 20 μ l volumes containing about 0.03 μ g DNA for 3 min at 37°, in 10 mM Tris-HCl pH 8.0-1 mM MgCl₂-50 mM NaCl-10 mM dithiothreitol with 1.5 U Exonuclease III (18), or in 0.05 M glycine-KOH pH 9.4-10 mM MgCl₂-10 mM dithiothreitol with 0.06 or 0.6 units of λ -Exonuclease (19).

Nick Translation

One microgram samples of DNA were labeled in 100 μ l incubations with ³H-TTP (New England Nuclear, Boston, MA) by nick translation (20). Labeled DNA was separated from the reaction mixture by chromatography on the Bio Gel A50M column described above. The DNA peak eluting with the exclusion volume was sonicated (4) in 0.12 M NaPO₄ pH 6.8, heat denatured, and stripped of foldback sequences on hydroxyapatite at 60°C. The specific activities achieved with this procedure ranged from 1-5 x 10⁷ cpm/ μ g.

Reassociation Experiments

All DNA was sonicated to about 400 bp (4). Reassociation experiments were carried out in 40-160 μ l incubation volumes as described (2,5). Reassociation curves were analyzed by least squares analysis using the computer program of Pearson, et al. (21).

RESULTS

DNA From Human Lymphocytes That Resists Alkali Denaturation

Nuclei from day 3 lymphocytes labeled with ³H-thymidine were lysed and the DNA was gently sheared as described in Materials and Methods. When the alkali denaturation-neutralization-phenol extraction method (13) was employed to remove alkali denatured DNA from that remaining double stranded, 1.2 - 2.0% of the ³H-thymidine labeled DNA was recovered in the "double-stranded" fraction. This would select for fragments containing large double stranded

regions, since short regions of internal complementarity would not prevent the otherwise single stranded fragments from partitioning in the phenol (13). However, in multiple experiments, the "double-stranded" DNA fraction contained no detectable closed circular duplex DNA when centrifuged to equilibrium on ethidium bromide-caesium chloride gradients (data not presented).

S₁ Nuclease Digestion

DNA in the "double-stranded" fraction was then tested for resistance to S₁ nuclease (Table I). Prior to a second denaturation step essentially all "alkali-resistant" DNA also resisted S₁ nuclease digestion; even after heat denaturation 47% remained S₁ resistant. In other experiments 98% of this DNA was solubilized by DNase I (data not presented). S₁ resistance was not

TABLE I

S₁ Nuclease Resistance After Sequential Alkali and Heat Denaturation

	Enzyme	Acid Precipitable cpm	
		³ H	¹⁴ C
1. ³ H Alkaline resistant	0	1570 (100%)	
2. ³ H Alkaline resistant	+	1545 (99%)	
3. ³ H Alkaline resistant + ¹⁴ C lymphocyte denatured	+	1448 (92%)	387 (4.6%)
4. ³ H Alkaline resistant, heat denatured + ¹⁴ C lymphocyte denatured	+	723 (47%)	404 (4.8%)
5. ¹⁴ C lymphocyte, native	0		6969 (100%)
6. ¹⁴ C lymphocyte, native	+		6549 (96%)
7. ¹⁴ C lymphocyte, denatured	0		8413 (100%)
8. ¹⁴ C lymphocyte, denatured	+		378 (4.5%)

Each incubation reaction contained, in 1.0 ml, 0.025 M potassium acetate pH 4.5 - 0.1 mM ZnSO₄ - 0.15 M NaCl, 20 µg denatured calf thymus DNA, and approximately 1 µg of either ³H-thymidine-labeled "alkaline-resistant" DNA or ¹⁴C-thymidine-labeled whole lymphocyte DNA, or both. "Enzyme" refers to 20 units of S₁ nuclease. DNA samples were denatured by heating at 100°C for 10 minutes prior to adding other components to the incubation vials. Incubations were carried out at 37°C for 30 minutes, then stopped by the addition of 50 µg salmon sperm DNA carrier and 10 ml 5% trichloroacetic acid. After 30 min at 0°C, the precipitates were collected on Unipore filters (Biorad), dissolved and counted as described (5). Numbers in parentheses represent the percentage of counts recovered relative to a control incubation in the absence of enzyme.

due to an enzyme inhibitor because about 95% of heat denatured ^{14}C -thymidine labeled whole cell lymphocyte DNA in the same incubation reaction was digested by the enzyme. This level of S_1 resistance in whole cell DNA is similar to that found by others (22-24), and is due to the presence of short inverted repetitive sequences. Since none of the ^3H -thymidine labeled DNA could be shown to be closed circular in structure, its high degree of S_1 resistance after two denaturation steps could be due either to inverted complementary sequences that can "snap back" to double-stranded form when the denaturing stimulus is removed or to internal cross-links. The latter possibility was excluded by electron microscopy.

Electron Microscopy of Alkali-Resistant DNA

Alkali-resistant DNA was heat denatured but not treated with S_1 nuclease, then spread and viewed by electron microscopy (Materials and Methods). Photographs were taken of a random sampling of DNA molecules and their contour lengths were calculated. There was no ordered pattern apparent in the lengths of 9 molecules measured: 30.8, 27.7, 19.9, 18.9, 18.1, 15.8, 14.5, 14.2, and 13.6 microns. The longest corresponded to about 93 kb but all were quite large considering that the previously described inverted repeats comprising 5% of human DNA are in the range of 190-300 bp (23,24). One hundred consecutive molecules were scored for the presence of single stranded regions; 71 appeared to be fully double stranded with blunt ends, 27 had one single-stranded tail at one end, while 1 had free single strands at both ends.

When large numbers of fragments were viewed, about 1% were found to have what appeared to be a clearly defined single-strand loop at one end (Fig. 2), and represent very large inverted complementary molecules. We considered the possibility that blunt-appearing ends on at least some of the other molecules might represent hairpin loops that were too short to be visualized. We therefore partially denatured the IR-DNA in order to generate more single-stranded regions (17). Since IR-DNA is derived from dispersed genome locations (see below), this process would be expected to randomly involve the ends and thereby expose more terminal hairpin loops if they were present. Of 50 consecutive molecules scored, 9 had well defined, apparently single-stranded loops at one end (data not presented). Thus it appeared that inverted complementary sequences might account for a substantial number of the fragments present in IR-DNA preparations.

The dispersed size of these fragments and the fragility of long single strands and their propensity to tangle during spreading technically

Figure 2 - Inverted-repetitive DNA: DNA fragments partitioning in the aqueous phase after two sequential rounds of alkali denaturation-neutralization-phenol extraction were examined by electron microscopy (Materials and Methods). Shown are two double-stranded molecules with terminal single-strand hairpin loops (arrows). Scale lines represent 1 micron (large pictures). The inset is an enlarged view of the adjacent loop.

prevented a comparison of double-stranded versus fully denatured length to prove that most were indeed inverted repeats. However, two other experiments support this hypothesis: [1] When IR-DNA was treated with either the 5'-Exonuclease from bacteriophage λ , or *E. coli* Exonuclease III, which selectively degrades 3' termini, only fragments with single-strand tails at one end were generated. There was no increase in the number of fragments with single-strand tails at both ends (Table II). The experiment utilized a blinded protocol to minimize observer bias during viewing (Experiment I, Table II). Although about half of the molecules remained double stranded at both ends in Experiment I, a second incubation utilizing 10-fold more enzyme decreased that fraction to 18% (Table II, Experiment II) without increasing the fraction with single-strand tails at both ends. This indicates that the opposite ends of the fragments lacked free 5' or 3' termini, presumably because they were, in fact, hairpin loops. We cannot exclude the possibility that these termini were "blocked" by the presence of tightly associated protein. However, the preparation used for the experiments presented in Figure 2 and Table II was incubated at 37°C with 0.5 mg/ml pronase in an SDS containing buffer for 18 hours prior to denaturation; it would be unusual for a protein to remain intact after this treatment. [2] In order to exclude cross-links as a possible trivial explanation for persistence of double stranded regions after two denaturation steps, the high molecular weight double stranded fragments eluting in the A50M column exclusion volume after S_1 nuclease digestion (see below) were studied by electron microscopy before and after heat denaturation. If the double stranded fragments resisted denaturation because they were cross-linked internally, S_1 nuclease might induce a few random nicks but should not remove the cross-link. Thus most molecules should maintain double stranded regions after another denaturation step. However, of 150 consecutive fragments scored, only 4 contained double stranded regions. When 22 fragments with double-stranded regions were identified, only 4 had single-stranded tails at both ends; these probably represent cross-linked fragments. The others might be cross-linked or might be incompletely digested inverted repeats. Otherwise these results indicate that S_1 nuclease removed a single stranded region responsible for most (>95%) of the population's original resistance to denaturation. This is strong indirect evidence that these fragments are comprised of inverted complementary sequences held together by a single-stranded hairpin loop that in most instances is too short to be visualized under electron microscopy.

TABLE II

3' or 5' Exonuclease Treatment of IR-DNA

EXPERIMENT	ENZYME ¹	OBSERVER ²	APPEARANCE OF DNA FRAGMENTS: ³		
			Number (percent)		
			D/D	D/S	S/S
[I]	Control	A	52	10	1
		B	$\frac{36}{88}$ (78)	$\frac{10}{20}$ (18)	$\frac{4}{5}$ (4)
	λ (.06 units)	A	39	52	4
		B	$\frac{22}{61}$ (42)	$\frac{25}{77}$ (53)	$\frac{3}{7}$ (5)
	Exo III	A	30	26	1
		B	$\frac{21}{51}$ (48)	$\frac{27}{53}$ (50)	$\frac{2}{3}$ (2)
[II]	λ (0.6 units)	A	12 (18)	50 (77)	3 (5)

The same preparation of IR-DNA as in Figures 2-4 was digested with a 3' or 5' Exonuclease as described in Materials and Methods.

1) λ = 5' Exonuclease from bacteriophage λ ; Exo III = 3' Exonuclease from E. coli; control incubations were performed in parallel in the λ Exonuclease buffer without enzyme.

2) In experiment [I], samples were prepared for viewing by observer A. The grids were then numbered by an outside observer and viewed separately by observers A and B without knowledge of the numbering system. Consecutive molecules for which both ends could be clearly identified were scored. After the scores were tabulated, the code was broken; scores for each sample as derived by each observer are presented. In experiment [II], the incubation was repeated with 10-fold more λ Exonuclease.

3) The letters indicate the appearance of each end of the fragments scored: D = double-stranded, S = single-stranded. Thus, D/D indicates both ends double-stranded.

Size of Inverted Repetitive Sequences

In order to assess the size distribution of high molecular weight fragments present in the ³H-thymidine-labeled alkali-resistant DNA, after heat denaturation a preparation pooled from two cultures was digested with S₁ nuclease, extracted with phenol:chloroform:isoamyl alcohol, and precipitated with ethanol. The precipitate was taken up in column buffer and applied to

a Bio Gel A50M column. (The column had been calibrated with ^3H -thymidine labeled high molecular weight cell DNA and ^{14}C -thymidine labeled cell DNA sonicated to a size of about 400 bp). Fifty-nine percent of the acid precipitable counts in this DNA preparation were resistant to S_1 nuclease digestion; 85% of the ^3H -thymidine eluted at the position of high molecular weight DNA (data not presented). In order to confirm that DNA fragments in the column exclusion volume remained very large, fractions containing the high molecular weight peak were pooled and concentrated with *n*-butanol; aliquots were then spread and viewed under electron microscopy. The contour lengths of 100 consecutive molecules were measured, yielding a size range from 0.15 - 20.95 microns. When the amount of DNA in each size class was calculated as a percent of the total, fragments larger than 1 micron (about 3030 bp) accounted for 94% of the DNA (data not presented).

These data indicate that very high molecular weight inverted repetitive sequences are synthesized in human lymphocytes during active growth. The quantity of these sequences present in the cells, although representing about 1-2% of the ^3H -thymidine incorporated, is small since the specific activity of IR-DNA is higher. For example, in a representative preparation the specific activity of purified cell DNA was 650 cpm/ μg , while the specific activity of DNA eluting in the high molecular weight fraction from the A50M column was 1550 cpm/ μg . These specific activity data suggest that high molecular weight IR-DNA is enriched in newly synthesized molecules, but the experiments do not assess quantitatively the relative proportion of molecules that might be carried over from resting lymphocytes.

Sequence Complexity of High Molecular Weight IR-DNA

In preliminary experiments, digests of high molecular weight IR-DNA with Hind III or EcoRI yielded smears on agarose gel electrophoresis that were indistinguishable from nuclear DNA digests. Thus IR-DNA appeared to be of high complexity. In order to document this more precisely, the sequence complexity of IR-DNA was studied by reassociation kinetic (C_{ot}) analysis. All of the following experiments utilized A50M high molecular weight IR-DNA fractions pooled from three separate culture preparations. The strategy was to drive the reassociation of nick translated ^3H -labeled IR-DNA with whole cell DNA from either resting lymphocytes or day 3 actively growing lymphocytes, and compare those data to the reassociation curve derived from nick translated ^3H -labeled whole cell DNA driven by whole cell DNA. As demonstrated in Figure 3, no difference could be detected among the three experiments. High molecular weight IR-DNA therefore represents sequences from es-

Figure 3 - Reassociation of high molecular weight IR-DNA driven by whole cell DNA: Nick translated ^3H -IR-DNA (Δ , $\square$) and nick translated ^3H -day 3 stimulated lymphocyte whole cell DNA ($\bullet$) were reassociated in the presence of unlabeled resting lymphocyte ($\bullet$, $\square$) or day 3 stimulated lymphocyte (Δ) DNA. The reassociation curves were fitted assuming 3 components by least squares analysis (17). The line drawn represents the computer solution for ^3H -cell DNA driven by cell DNA ($\bullet$); the other curves could not be distinguished from this solution.

entially the whole of the genome.

The fact that IR-DNA quantitatively represented only 1% of nuclear DNA but contained a distribution of sequence classes indistinguishable from the whole of the genome suggested that inverted repetitive structures might be present in different locations in different cells. This would be part of the original hypothesis: individual cells might generate duplications of dispersed regions of their DNA, a process that would result in "amplification" of a large portion of a genome when DNA from the entire population of cells was analyzed. In order to further examine this possibility, released DNA and IR-DNA were compared.

Relationship of High Molecular Weight Nuclear IR-DNA to Released DNA

First we determined whether released DNA contained a large proportion of high molecular weight inverted repetitive sequences. Using the criterion of exclusion from phenol after the standard alkali denaturation-neutralization step, in five different released DNA preparations the proportion of partitioning as double-stranded sequences ranged from 14-25%. This was substantially higher than the 1.2-2.0% observed for lysates of cells harvested on day 3. In order to determine whether there is a quantitative relationship between the fraction present as IR-DNA in nuclei and that which is released by trypsin, cells labeled with ^3H -thymidine were harvested during active growth (day 3), at the onset of the growth plateau (day 4), and after cessa-

tion of growth (day 5) (5,6). DNA release is first observed on day 4 (5-7). The fraction of labeled DNA partitioning as double-stranded after denaturation was measured in nuclear lysates from untreated cells, and on days 4 and 5, from cells after treatment with 1 $\mu\text{g}/\text{ml}$ trypsin for 5 minutes; these results were compared to the same fraction in the trypsin-released DNA (Table III). The fraction of large foldback sequences present in nuclear lysates increased from 1.8% on day 3 to 4.2% on day 5; after trypsin treatment that day 5 fraction was decreased to 2.2%. In contrast about 16% of the two trypsin-released preparations partitioned as large double-stranded sequences after denaturation, and this fraction was appropriate for the amount lost from the cells: on day 5 13.8% of the acid precipitable ^3H -thymidine was

TABLE III

Quantitation of Foldback Sequences Present in Cell DNA and Released DNA from the Same Culture

Day of Culture	Cell Treatment (Trypsin)	Acid Precipitable CPM			Released DNA (5 ml)		
		Cell DNA (2ml) Before Phenol	Cell DNA (2ml) After Phenol	%	Before Phenol	After Phenol	%
3	0	17905	339	1.8%			
		18264	321				
4	0	19416	429	2.2%			
		19840	418				
4	+	21030	263	1.5%	2940	558	16.0%
		14630	262		2697	345	
5	0	18398	838	4.2%			
		19634	757				
5	+	15250	347	2.2%	4968	845	16.5%
		16537	338		4755	760	

Stimulated lymphocytes from a single donor were cultured in 50 ml flasks at an initial density of $0.5 \times 10^6/\text{ml}$. Cells were harvested during active growth (day 3) and on days 4 and 5 when cessation of cell growth occurs and DNA release is elicited (5-7). A single flask was used for each "no treatment" set, while two flasks were harvested for the trypsin treated sets. Cells from all sets were washed twice with warm HBSS made without NaHCO_3 (5). For DNA release, cells from each flask were resuspended in 25 ml HBSS without NaHCO_3 ; an aliquot was removed from each for determination of total cellular acid precipitable ^3H -thymidine, and the cells were treated with 1 $\mu\text{g}/\text{ml}$ trypsin at 37°C for 5 min. Soybean trypsin inhibitor was added to 25 $\mu\text{g}/\text{ml}$ and the cells were pelleted at $2000 \times g$ for 5 min. Nuclei were prepared from the cell pellets as usual. The incubation medium was made up to .01 EDTA - .01 M Tris-HCl pH 7.4 - .6% SDS and centrifuged at $10,000 \times g$ for 10 min. Trypsin-released acid precipitable ^3H -thymidine represented 7.2% and 13.8% of the total cellular CPM of days 4 and 5 respectively.

released by trypsin, while 16.5% of that represented large double-stranded sequences. Therefore about $.138 \times .165 = .023$ of the cell DNA was accounted for in this preparation.

We assume that the fraction of released DNA partitioning as double-stranded after denaturation represents IR-DNA, but this assignment is tentative because these preparations have not been subjected to the same characterization described above for nuclear IR-DNA.

Comparison of Released DNA and Nuclear IR-DNA by Reassociation Analysis

The similarity of nick translated ^3H -labeled IR-DNA to released DNA was assessed by driving the reassociation of IR-DNA with large quantities of released DNA. Each IR-DNA incubation was performed in parallel simultaneously with the reassociation of ^3H -labeled nick translated whole cell DNA driven by an identical amount of released DNA serving as the control. Three different preparations of released DNA were tested; the amounts of released DNA purified from the three cultures varied, but were all relatively small (5) so that the number of data points in the high C_0t range were limited. Each released DNA preparation was incubated in at least two different concentrations with both ^3H -IR-DNA and ^3H -cell DNA; one extra concentration was utilized with ^3H -cell DNA incubations in the experiments in Figure 4A and B. In addition, to ensure that reagents would not contribute an artefact to the experiments, two different nick translated probes were made from the IR-DNA and cell DNA preparations and were used with each released DNA preparation. The results are presented in Figure 4.

Where final reassociation values could be predicted from computer curve fitting (Figures 4A and B), the values were similar for both ^3H -IR-DNA and ^3H -cell DNA. This indicates that the overall complexity of IR-DNA is similar to that for cell DNA as a whole and supports the concept that IR-DNA may be derived from random locations in the genome. However, these terminal reassociation values were substantially less than achieved when these probes were incubated with cell DNA (Figure 3) because the total sequence complexity of released DNA is limited relative to the whole genome (5).

The important difference present in each pair of curves involves 10-15% of the ^3H -IR-DNA probe. This fraction reassociated as a component with a $C_0t_{1/2}$ of 1.3 and 1.06 M s/l in Figures 4A and B, respectively; in Figure 4C the same fraction appeared to be present but a computer fit was not possible since the small amount of released DNA did not permit higher C_0t values. This component was not detectable in the reassociation curves for the ^3H -cell probes. Although the difference between the ^3H -IR-DNA and ^3H -cell DNA

Figure 4 - Reassociation of IR-DNA and whole cell DNA in the presence of released DNA: open symbols with the solid line in each represent ^3H -day 3 lymphocyte whole cell DNA, while closed symbols with the dashed line represent ^3H -IR-DNA. These are the same probes used in Figure 5. Similar symbols in each panel (e.g., $\bullet$, $\circ$) represent simultaneous incubations done in parallel with an identical amount of released DNA present for each of the two probes. Different symbols (e.g., $\bullet$, $\square$, $\triangle$) represent separate incubations with different concentrations of released DNA. Each panel presents the results obtained from one preparation of released DNA. Only these three consecutive preparations of released DNA were studied. Curves were fitted to (A) and (B) by computer analysis; in (C), straight lines were drawn for continuity. In (A), the inset expands the ordinate so that points from different incubations can be designated.

curves was small it was highly reproducible in each of the separate incubations and was defined by a large number of data points. These experiments demonstrate that released DNA preparations contain sequences that will drive the reassociation of both whole cell DNA and IR-DNA probes. However, 10-15% of the IR-DNA probes are represented by sequences that cannot be detected as such in the cell DNA probes and therefore appear to be specifically enriched in IR-DNA. These sequences are abundant in released DNA; they are present about 400 times more frequently than high complexity unique sequence elements. Therefore a portion of high molecular weight IR-DNA and released DNA

is directly related; this is further evident that released DNA might be derived from high molecular weight IR-DNA precursors. In other reassociation experiments (data not presented), the quantity of mitochondrial DNA present in released DNA was found to be less than 1 part in 1770, thus excluding mitochondrial DNA contamination as a cause for the IR-DNA-released DNA shared component.

DISCUSSION

The data presented above document the existence of high molecular weight inverted repetitive sequences in newly synthesized nuclear DNA and indicate that they may be unstable. Our initial model to explain their presence (Figure 1) was chosen because of its possible relevance to amplification but remains only an hypothesis since we have no direct evidence to support its validity in this system. However, results from other systems provide precedent either directly or indirectly for randomly generated, high molecular weight inverted complementary structures in both prokaryotic and mammalian DNA.

Alberts and Doty (16) demonstrated that after heat, alkali, or formamide denaturation, about 6% of DNA molecules extracted from B. subtilis spontaneously renatured when the denaturing conditions were removed; renaturation was assessed by specific transforming ability as well as physical behavior, including partitioning in a dextran-polyethylene glycol two phase system. Using the latter criterion, they found that about 1% of calf thymus DNA behaved in a similar manner (16). Sedimentation studies of renatured B. subtilis DNA indicated that the renatured strands were joined near the end of the double stranded molecules (16). Further work demonstrated that these "cross-links" occurred at random along the genetic map (25). Interestingly, the presence of cross-links appeared to relate to the size of the original molecule, since it was not observed with λ phage DNA, but was detectable in SP82 phage DNA (MW = 130×10^6 daltons) at about 25% of the frequency found in B. subtilis or calf thymus DNA (25). The author suggested that these cross-links might be formed when very large DNA molecules were sheared (25).

However, Chow, et al. (26) provided evidence that large inverted repetitive molecules can result from illegitimate recombination or abnormal replication. They studied rare deletion mutants of phage λ , called λ_{dv} plasmids, that retain the DNA replication genes and exist as covalently closed circular DNA molecules (27). One class of these plasmids was comprised of monomers or tandemly repeated oligomers, while the other equally

frequent class was comprised of inverted repetitive sequences as judged by electron microscopy of denatured nicked circular plasmid molecules and by heteroduplex analysis with intact phage λ DNA (26). Some of the latter class of mutants lacked any detectable unique sequences so that no single strand hairpin loop could be detected at the ends of the snap back molecules, an appearance similar to the molecules presented in this paper. The authors' models to explain the origin of these structures all involved joining of parental to parental and progeny to progeny strands of a replicating λ molecule at the two replication forks (26). It is relevant to note that ligation of the free double stranded fragments in the model presented here (Figure 1) would accomplish the same thing but, in addition, this model would allow the persistence of an intact replicated chromosome.

Other authors have demonstrated what they interpreted to be cross-linked molecules in mammalian cells. Rommelaere and Miller-Faures (28) labeled Chinese hamster cell DNA with bromodeoxyuridine for one or less generation, then analyzed its density profile in alkaline cesium chloride gradients. About 1.5% banded at an intermediate, light-heavy density (28). This figure is similar to the amount of high molecular weight IR-DNA isolated from growing lymphocytes, but the data presented here demonstrate that internal cross-links could account for only a small portion of this DNA. The complementary strands of most IR-DNA molecules are attached by a terminal S₁ nuclease-sensitive site.

This leads to the same question raised by Albert's findings (25), namely whether such structures are due to artificial cross-links associated with the shearing of large molecules or whether they might be associated with recombination or abnormal replication. The facts that the fraction of high molecular weight IR-DNA appears to increase in cells between days 3 and 5 of culture (Table III), and that released DNA is substantially enriched in sequences that resist denaturation, presumably IR-DNA, argue that their origin is dependent upon some cellular mechanism rather than simply being the unusual product of shear during isolation. In addition, other work indicates that the formation of similar structures may be increased by agents that interfere with DNA replication.

Woodcock, *et al.* (29,30) developed a system to identify reinitiation of DNA synthesis in DNA segments that had already been replicated earlier in the same S phase. In this system, partially synchronized tissue culture cells of human origin were first pulse labeled for 30 min with [³H]deoxycytidine, then the label was chased with a large excess of unlabeled deoxy-

cytidine for 90 min. The cells were then divided into two portions. Half were exposed to cytosine arabinoside (29) or cycloheximide or adenine arabinoside (30) for one hour followed by a density labeling period with BrdUrd in the presence of FdUrd and deoxycytidine. The other half, control cells, were not exposed to an inhibitor but were immediately placed in density labeling medium. DNA was purified and centrifuged to equilibrium in neutral CsCl gradients, and the region containing light-heavy DNA was quantitated, then recentrifuged in alkaline CsCl. Molecules in which the ^3H and BrdUrd density labels coexisted either on the same strand or as cross-linked strands could not be distinguished in this system, since on the alkaline gradients the two could coincide; in control cells essentially all of the ^3H label was present at the density of the BrdUrd substituted strands (29). However, in DNA from cells exposed to any of the three inhibitors, as much as 50% of the ^3H label was present at the density of the unsubstituted strand (29,30). Other control experiments appear to exclude repair synthesis as a cause for this observation. The authors conclude that this finding is likely due to reinitiation of DNA synthesis (identified with the BrdUrd label) in an already partially replicated region (identified by the ^3H label) and their model (29) to explain this is similar to that presented here.

The enrichment of foldback sequences in released DNA and the sharing of a set of repetitive sequences by high molecular weight IR-DNA and released DNA indicate that released DNA may be derived at least in part from such IR-DNA structures. Since the sequence complexity of released DNA is less than that of both whole cell DNA and IR-DNA (Figure 5), it may represent a selected set of IR-DNA sequences, but this might simply reflect limited degradation. The model presented here would explain the previously observed amplification of released DNA sequences during lymphocyte growth (2), since even a single extra round of replication at participating sites would double the quantity of involved sequences relative to normally replicated regions. These data support the hypothesis (2) that dispersed amplification is a property of normal diploid human cells in tissue culture. Whether it occurs in vivo remains to be tested.

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1. Abbreviations: PHA, phytohemagglutinin; bp, nucleotide base pairs; kb, kilobase pairs; IR-DNA, inverted repetitive DNA; mt-DNA, mitochondrial DNA; C_{ot} , concentration of DNA x time expressed as moles of nucleotides per liter x seconds; $C_{ot}1/2$, C_{ot} value at which reassociation is half complete.
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