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Replication of Latent Epstein-Barr Virus Genomes in Raji Cells

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The replication of the 50 to 60 latent, predominantly extrachromosomal, Epstein-Barr virus genomes maintained by the Burkitt-lymphoma-derived Raji cell line was investigated by using a Meselson-Stahl density transfer approach. Samples of DNA isolated from cells cultivated for different periods in bromodeoxyuridine-supplemented medium were fractionated according to density, and the distribution of viral and cellular DNAs among the heavy-, hybrid-, and light-density species was quantitated. The results indicate that the majority of latent Epstein-Barr virus DNA plasmids each replicate once during the cell cycle.

Nearly all of the human, B-type, lymphoid cell lines established in continuous culture maintain a characteristic number of latent Epstein-Barr virus (EBV) genomes (22, 34), which in the case of Raji is 50 to 60 per cell (21, 23). The majority of these viral genomes are carried as free, extrachromosomal DNA plasmids (1, 20), although the possibility of one or more integrated copies of the complete EBV genome cannot be excluded (2, 3, 15). The stable retention of a constant number of EBV genomes in actively dividing cells cannot be explained simply by the semiconservative replication of viral DNA as an integral part of the host chromosome DNA.

The amount of EBV DNA has been reported to double early during the cellular S phase (8), and the finding of a few theta-structured EBV genomes at this time suggests that the plasmid forms can act as independent replicons (6). However, whether each EBV plasmid replicates once during the cell cycle or whether only a few viral genomes act as templates for the synthesis of many progeny molecules was not established. In the present study, a Meselson-Stahl density transfer approach (17) was used to determine if most or only a few of the EBV DNA plasmids function as templates for the maintenance of a constant number of viral genomes in the Raji cell line.

Growth of Raji cells in BUdR-supplemented medium. Tumor-derived lymphoid cell lines proceed through several cell doublings in medium that is supplemented with 30 μM bromodeoxyuridine (BUdR), and it has been reported that it is possible to recover DNA in which 90% of the thymidine residues are replaced with the heavy-density base analog (9). Mycoplasma-free Raji cells were cultured in RPMI 1640 media containing 8% fetal calf serum. The cells were subcultured every second day for 8 days before the addition of BUdR to maintain the asynchronous culture in continuous log phase. [^{3}H]BUdR (13 mCi/mol; 5-bromo-2′-deoxy[1′,2′^{3}H]uridine; Amersham Corp.) was then added to a final concentration of 30 μM and growth of the culture, which was incubated in the dark in foil-wrapped flasks, was periodically monitored. In agreement with previously reported results (9), both the control and BUdR-treated cultures proceeded through three population doublings at near logarithmic expansion with a generation time of approximately 23 h.

Higher concentrations of halogenated pyrimidines are known to induce lytic EBV replication in nonproducer cells like Raji. To preclude or estimate the contribution of lytic EBV DNA synthesis, the number of cells in which the productive viral cycle was induced was determined by direct immunofluorescence of acetone-fixed cell smears with a fluorescein isothiocyanate-conjugated Burkitt lymphoma serum of high early antigen (EA) titer (12). No EA-positive cells (>0.02%) were seen in any of the batches of BUdR-treated cells at the time of DNA isolation. In the positive immunofluorescence control, consisting of Raji cells exposed to 50 μg of iododeoxyuridine per ml for 2 days, 1% EA-positive cells were observed. It is concluded that lytic EBV replication was not induced in any of the cell samples analyzed.

Isolation and analysis of BUdR-labeled DNA. Total DNA was isolated from Raji cells which had been cultivated for 12, 36, 48, 66, and 96 h in BUdR-containing medium. The DNA was freed from RNA and proteins by standard methods, sheared to a mean molecular weight of 10×10^6, and fractionated by equilibrium density gradient centrifugation (2). All operations were performed in subdued light to minimize light-induced degradation of the BUdR-containing DNA (29).

A typical DNA separation is shown for the 48-h sample in Fig. 1. Three peaks of A_{260}-absorbing material are seen at densities of 1.8, 1.75, and 1.7 g/cm^3. Isotope labeling was associated only with the two high-density peaks (Fig. 1A), with the mean specific radioactivity of the DNA in fractions 13 to 20 being twice that of the material in fractions 21 to 29 (7.0×10^3 cpm/μg of DNA versus 3.6×10^8 cpm/μg). The specific activity varied among individual fractions because some separation of DNA on the basis of G+C content was achieved within each peak. If it is assumed that BUdR is incorporated only during semiconservative DNA replication, the hybrid (heavy-light; H-L)-density peak at 1.75 g/cm^3 consisted of an unmarked template strand and a BUdR-substituted daughter strand, while the thymidine residues of both the template and the newly synthesized DNA strands of the density peak at 1.8 g/cm^3 (heavy-heavy; H-H) were substituted with the brominated base analog. Note that as expected no label was associated with the unreplicated light-light (L-L)-density peak at 1.70 g/cm^3.

For each of the five DNA preparations analyzed, the distribution of EBV DNA associated with the H-H-, H-L-, and L-L-density peaks was quantitated by nucleic acid hybridization of individual CsCl gradient fractions wth ^{32}P-labeled EBV cRNA (16). The results for the DNA sample isolated at 48 h are shown in Fig. 1B. The density shifts produced by incorporation of BUdR in place of thymidine in one and both strands of EBV DNA were not of the same magnitude as those seen with the bulk DNA. The difference is attributed to the higher proportion of G · C base pairs (bp)

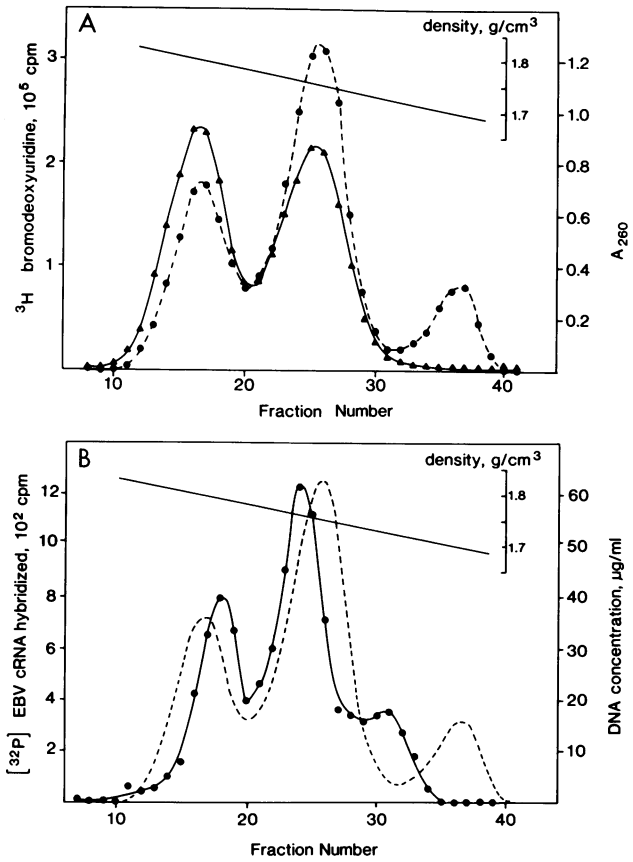

FIG. 1. Cesium chloride density gradient separation of DNA from cells exposed to BUdR for 48 h. (A) The distribution of total DNA (●) was determined from A_{260} measurements made on individual fractions, and the BUdR-labeled material (▲) was located by counting a small volume of each fraction for the tritium isotope. The density of the CsCl solution over the midregion of the gradient was calculated from refractive-index readings made on selected fractions immediately after the gradient was collected. (B) The location and the amount of EBV DNA (●) were deteremined by hybridizing 150-μl samples of individual gradient fractions with ^{32}P-labeled EBV cRNA. The distribution of total DNA (- - -) is expressed as micrograms per milliliter.

(58%) in EBV DNA (27, 30), which results in proportionally fewer thymidine residues being replaced with the density label. Thus, while the unsubstituted form of EBV was 20 mg/cm^3 more dense than unsubstituted host cell DNA in CsCl solution, the fully substituted EBV DNA was almost 10 mg/cm^3 lighter than its H-H-density host DNA counterpart. While separation among the various EBV DNA forms was not as great as for the bulk of the DNA, it was still possible to estimate what fraction of viral DNA was associated with the various forms. To compare the amount of EBV DNA associated with the H-H, H-L, and L-L peaks of cellular DNA, it is necessary to define the densities of the same form of the two DNAs slightly differently.

Mode of latent EBV genome replication. From graphs similar to that shown in Fig. 1, the relative proportions of total DNA and EBV-specific DNA associated with their respective H-H-, H-L-, and L-L-density peaks were calculated. Data for each of the five DNA preparations analyzed are summarized in Fig. 2. The relative amount of unreplicated L-L-density DNA decreased with time, while the proportion of H-H-density DNA, which in the case of host DNA contains single DNA strands synthesized in two different S phases, increased with time. Within experimental error, the distribution of EBV DNA among the three different viral density species was the same as that of the bulk DNA. Thus, in agreement with the immunofluorescence data, there was no excess of H-H-density EBV DNA indicative of lytic viral replication having been induced in the BUdR-treated cells at any time during the 96-h experiment.

The relative amount of viral DNA associated with any of the three density species generated by semiconservative DNA replication in the presence of BUdR closely parallels what is seen with total DNA (Fig. 2). Had only a few viral genomes acted as templates for the formation of several progeny molecules, H-H-density material should have accumulated quite early after the addition of BUdR. For example, after one cell doubling, equivalent amounts of H-H- and L-L-density DNAs would be predicted, with the total amount of H-L-density DNA being only twice that of the number of genomes that were initially used as master templates (32). That this was not the case is clearly seen with the data collected on the cell sample isolated 12 h after the addition of BUdR, when no cell should have had an opportunity to experience more than a single S phase. Only 30% of the cells in the asynchronous culture had divided at 12 h, and as expected, no measurable amount of H-H-density cellular DNA was found. The hybridization data indicate that one-third of the viral DNA was duplicated once to form only H-L-density material at this time (Fig. 2); this result, within experimental error, is consistent with the amount of total DNA replication seen (38%).

Different modes of semiconservative DNA replication have been observed for extrachromosomal DNA elements in eucaryotic cells. Thus, some plasmids, such as the 2μm DNA circles of yeast, each replicate in a highly controlled manner only once during the S phase of the cell cycle (32). In contrast, other multicopy extrachromosomal DNAs, such as the rRNA genes of *Physarum* spp. (19, 28, 33) and the mitrochondrial DNA of mice (4), replicate at random

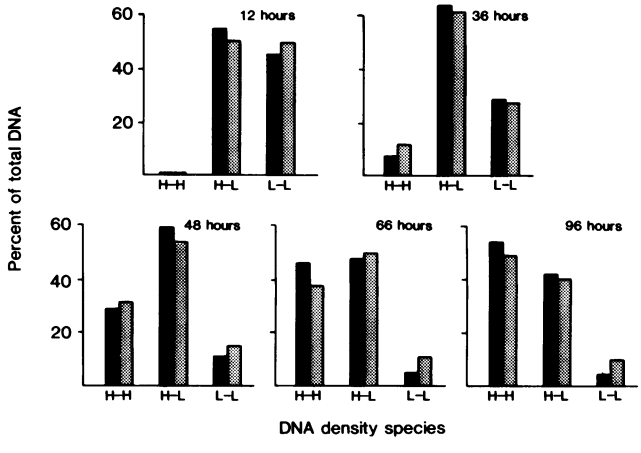

FIG. 2. Comparison of the amounts of EBV and cellular DNAs associated with each of the different DNA density species. The amounts of EBV and cellular DNAs associated with H-H-, H-L-, and L-L-density peaks were calculated from CsCl density gradient profiles similar to those shown in Fig. 1. The amounts of the three density species of DNA that were isolated from cells incubated for various periods in the presence of 30 μM BUdR are plotted. ■, Total DNA; ▩, EBV DNA. The times at which the DNA was isolated are indicated.

throughout much of the cell cycle. While some degree of random template utilization is not absolutely precluded by the results summarized in Fig. 2, the temporal order of replication of the majority of the latent EBV genomes resembles most closely that of the small yeast plasmid.

Effect of BUdR incorporation on the function of latent EBV genomes. Induction of the lytic EBV cycle by halogenated thymidine analogs requires continued DNA synthesis (5, 7, 11), and it has been hypothesized that the altered gene expression is the direct result of the incorporation of BUdR or iododeoxyuridine into DNA. Substitution of thymidine residues with halogenated derivatives is known to alter the thermal stability of DNA (13), binding of regulatory proteins (14), and gene expression in eucaryotic cells (26). The ability to isolate highly substituted EBV DNA in the absence of induction would, however, tend to argue against simple alteration of regulatory protein binding as the mechanism of BUdR activation of lytic functions in Raji cells. The mechanism by which 3- to 10-times-higher concentrations of BUdR induce early lytic functions in a few percent of the cultured cells is not clear.

The flow of viral DNA from the H-L- to H-H-density species paralleled that of cellular DNA (Fig. 2), and BUdR substitution in at least one of the two DNA strands did not obviously alter the duplication of latent EBV genomes. In this regard, it is noted that a potential origin for EBV DNA synthesis, defined by the ability of two noncontiguous sequences, mapping to the *Bam*HI C fragment of the B95-8 virus isolate, to provide the *cis*-acting fuction required for the replication of small recombinant DNA plasmids in EBV-nuclear-antigen (EBNA 1)-positive cells (31), is particularly rich in just A · T bp (10). Both sequences (the longer consists of about 20 copies of a 30-bp repeat unit that is only 33% G+C, and the shorter, 43% G+C, is a unit of 114 bp that includes a 65-bp region of diad symmetry) specifically bind a truncated EBNA 1 protein (24), and EBNA binding has been proposed as a mechanism for the maintenance of a constant number of EBV genomes in latently infected cells. The binding of EBNA to the 30-bp repeat unit has been found to modify the expression of adjacent genes of recombinant DNA plasmids (25). The apparent insensitivity of latent EBV DNA replication to BUdR incorporation observed here might be due to the use of Burkitt-lymphoma-derived Raji cells. The DNA used in both the construction of the small recombinant DNA plasmids and the DNA sequencing studies was derived from virus originating from a normal lymphoblastoid cell line established from an infectious-mononucleosis patient (18). In contrast to the Burkitt-derived lymphoma cell lines, normal lymphoblastoid cells such as the 883L line, from which the B95-8 virus was isolated, only proceed through a single cell cycle in BUdR-supplemented medium (9). The effect of BUdR incorporation on latent EBV DNA replication in lymphoblastoid cells has not, to my knowledge, been investigated.

The mycoplasma-free Raji cells and fluorescein isothiocyanate-conjugated antiserum with high EA titer were obtained from G. Klein.

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