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# Immortalization of Human T Lymphocytes After Transfection of Epstein-Barr Virus DNA

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**Epstein-Barr virus (EBV), a ubiquitous human herpesvirus, has the ability to transform human B lymphocytes. No other cell type has been experimentally transformed by EBV, either by intact virions or naked viral DNA and subgenomic fragments. Two immortalized human T-lymphoblastoid cell lines have now been established by transfecting cord blood lymphocytes with purified B95-8 viral DNA enclosed in fusogenic Sendai virus envelopes (RSVE) and then exposing the cells to EBV from a P3HR-1 cell subclone. One of these lines, which has been fully characterized, is termed HBD-1. This line is positive for EBV DNA and expresses surface OKT11, OKT4, and Tac receptors, but not M-1, μ immunoglobulin chains, EBV receptors, or B-1 surface markers. The cells contain fully rearranged T-cell receptor genes and germline immunoglobulin genes. The karyotype of the cells is normal, they do not require interleukin-2 for growth, and do not contain human T-lymphotropic virus type I. However, the HBD-1 cells contain incomplete EBV genomes and express several EBV-determined antigens, including the early antigen type D, membrane antigens, but not EBV-determined nuclear antigen (EBNA). This association of the EBV genome with permanently growing hematopoietic cells of non B-cell lineage should prove useful in studies on the mechanism of EBV-mediated cell transformation.**

EPSTEIN-BARR VIRUS (EBV) IS A HUman herpesvirus that causes infectious mononucleosis and is believed to be etiologically associated with Burkitt's lymphoma and nasopharyngeal carcinoma (*1*). One of the major distinguishing features of EBV has been its inability to infect any cells other than human B lymphocytes. Only mature B lymphocytes expressing specific viral receptors have been infected by the virus in vitro (*2*). The infection immortalizes the cells and the resulting lymphoblastoid cell lines carry latent viral genomes, but do not support virus replication. The latently infected cells express EBV-determined nuclear antigens (EBNA) that are considered to be the first products of the viral cycle and to be important for cell transformation. However, we have now established two EBV DNA-positive but EBNA-negative human T-lymphoblastoid cell lines. These cell lines were obtained after cord blood lymphocytes (CBL) were transfected with purified B95-8 viral DNA enclosed in fusogenic Sendai virus envelopes and then exposed to EBV from a P3HR-1 cell subclone. The cells were negative for retrovirus, had normal karyotypes, and expressed several antigens associated with the EBV replicative cycle. The lack of EBNA expression suggests that this antigen might not be an essential indicator of EBV infection. The demonstration that EBV genomes can be maintained indefinitely in human T lymphocytes may have important implications with regard to the role of EBV in the pathogenesis of certain human diseases.

The EBV genome-positive T-cell lines were established when we were attempting to map the regions of the EBV genome involved in B lymphocyte immortalization (*3*). While studying the expression of cloned EBV DNA fragments in freshly isolated CBL, we found that the EBV DNA region located within the Bam HI–D1 fragment stimulated cell growth but did not induce EBNA or cell immortalization (*3*). Because this region contains sequences deleted in the nontransforming P3HR-1 (P-EBV) virus DNA (*4*), we investigated whether freshly isolated lymphocytes could be transformed by concomitant exposure to P3HR-1 virus and either purified EBV DNA or the cloned Bam HI–D1 region that is missing from the P-EBV genome. We introduced the EBV DNA into CBL by a recently developed technique in which gene transfer is mediated by reconstituted Sendai virus envelopes (RSVE) (*3, 5, 6*). The DNA was encapsulated in fusogenic Sendai virus envelopes during envelope reconstitution. The DNA-containing RSVE were then fused with target cells and exposed to P3HR-1 virus of the HH514-16 strain (HH-EBV) (*6*). We chose this viral isolate because it was shown to induce little or no EBV-determined early antigen (EA) upon superinfection of Raji cells (*7*) and thus could be expected to be less cytotoxic than the parent P3HR-1 strain. The DNA-transfected, HH-EBV–exposed cells were cultured on an irradiated feeder layer of normal human fibroblasts (from explanted tonsil tissue) at 37°C in 5% $CO_2$ and tested weekly for EBNA induction and morphologic transformation. No EBNA induction or transformation was observed in cells coinfected with Bam HI–D1 fragment and HH-EBV. In contrast, cells transfected with purified EBV DNA from the transforming B95-8 strain and exposed to HH-EBV rapidly proliferated, then declined to few living cells about 4 weeks after infection, and finally grew out into a permanently transformed cell line about 2 months from the beginning of the experiment. As in previous experiments (*3*), CBL treated with RSVE/EBV DNA alone were transiently growth-stimulated and died after 4 to 5

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weeks. The cells exposed to HH-EBV alone died within 2 weeks after viral infection.

The properties of one of the cell lines, termed HBD-1, that was established by this procedure and has been grown continuously in culture for almost 2 years are summarized in Table 1. The HBD-1 line contained predominantly $OKT11^+$, $OKT4^+$, and $Tac^+$ lymphocytes, indicating their T-cell origin. The clonal T-cell nature of the HBD-1 line was confirmed by analysis of the configuration of the T-cell receptor gene [$\beta$-chain; see (*8*)]. Figure 1, A and B, shows results of probing HBD-1 cell DNA with CT$\beta$2 Jurkat complementary DNA (cDNA). Placental DNA (lanes d) provides the germline pattern with two germline bands of 12 and 4.2 kb in Eco RI digests (A) or 24 kb in Bam HI digests (B). The absence of germline bands in the HBD-1 sample indicates complete rearrangement of the T-cell receptor gene and demonstrates the mature differentiated T-cell character of the HBD-1 line. This was further confirmed by demonstrating a complete germline configuration in DNA probed with an immunoglobulin gene (*9*) probe ($J_H$) (Fig. 1, C and D).

Although HBD-1 cells expressed receptors for T-cell growth factor (interleukin-2; IL-2)(*10*), as detected by reactivity with

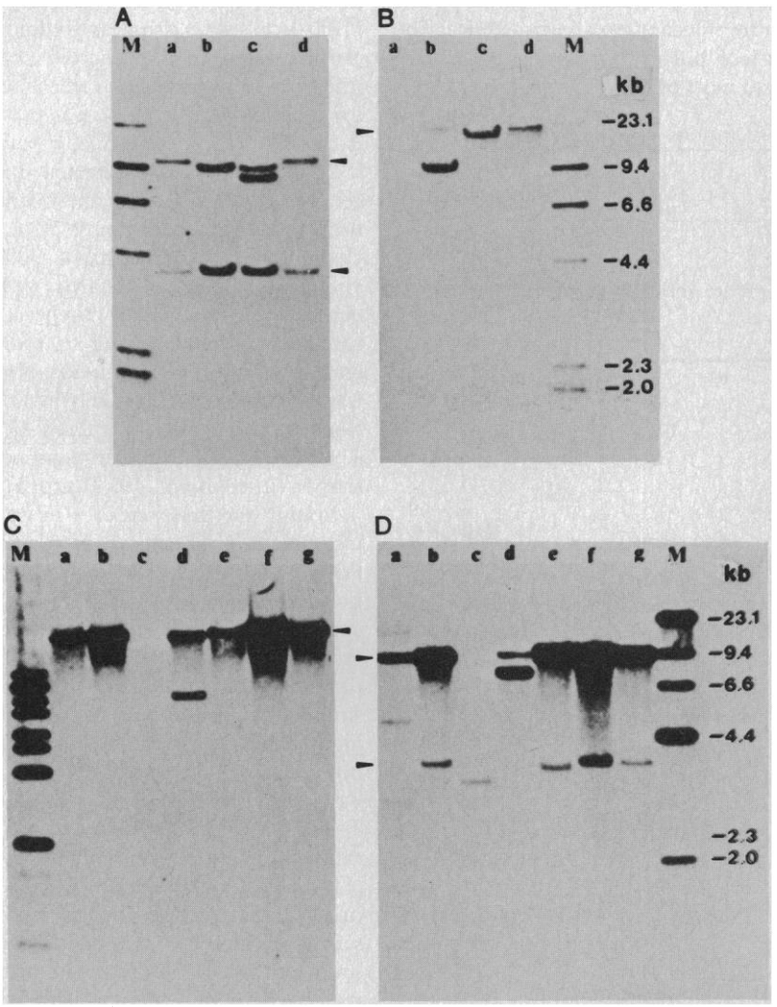


Fig. 1. Southern blot analysis of T-cell receptor gene and immunoglobulin H chain in DNA from HBD-1 cell line. HBD-1 cell DNA and control DNA from thymic leukemia cell lines CEM and 1301, Burkitt's lymphoma cells Raji and HH514-16 (subclone of P3HR-1), American Burkitt's lymphoma line Loukes, and placenta were analyzed with (A and B) a 770-bp human β-chain cDNA (*8*) or (C and D) a 5.6-kb Bam HI–Hind III $J_H$ probe (*9*). (A and B) Eco RI (A) or Bam HI (B) digests of DNA from Raji (a), CEM (b), HBD-1 (c), and placenta (d); (C and D) Eco RI (C) and Hind III (D) digests of DNA and Loukes (a), 1301 (b), HH514 (c), Raji (d), HBD-1 (e), CEM (f), human placenta (g). Arrows indicate germline positions as determined with placental DNA. Lane M denotes end-labeled Hind III digests with relative molecular weights in the margins. The sizes of the CTβ-germline restriction fragments obtained from placental DNA were 12 and 4.2 kb (Eco RI digest; A, lane d) and 24 kb (Bam HI digest; B, lane d). The germline fragment of 12 kb (Eco RI digest) was replaced in the HBD-1 line by the bands of 9.2 and 8.0 kb representing the rearranged CTβ gene (A, lane c). Similarly, the 24-kb germline fragment (Bam HI digest) was replaced in HBD cells by a 21-kb fragment (B, lane c). DNA was extracted by the guanidium isothiocyanate procedure (*26*). Samples of restriction endonuclease digested DNA (10 μg) were electrophoresed in a 0.8% agarose gel and blotted onto nylon membranes. The blots were hybridized to nick-translated CTβ or $J_H$ probe at 42°C in 50% formamide, 5× SSC (standard saline citrate), 3× Denhardt's solution, 1% SDS, and 100 μg/ml of salmon sperm DNA. Blots were washed at 55°C in 0.1× SSC and 0.1% SDS.

Table 1. Summary of principal features of the HBD-1 cell line. The HBD-1 cell line was analyzed for cell surface phenotype by means of an Ortho system 50H cytofluorograph (FACS). The monoclonal cell surface markers used were as follows: OKT3, OKT4, OKT11, and OKT8 (Ortho); B-1 (Coulter Electronics); and immunoglobulin M μ (IgM μ), M-1 (Tago). EBV receptors (EBV-R) and HTLV receptors (HTLV-R) were assayed by using fluorescein isothiocyanate-labeled virions and flow cytometry as described (*23*). EBV membrane antigen (EBV-MA) was detected by flow cytometry with mouse monoclonal antibody to MA. EBNA was assayed by an anticomplement immunofluorescence (ACIF) test (*24*) and Western blot. EA, VCA, and ATLA were determined by indirect immunofluorescence (IF) with the use of monoclonal antibodies to EA and VCA (Biotech Research Laboratories) or serum from an ATL patient, respectively. Poly(A)-dependent DNA polymerase activity (reverse transcriptase assay, RT) was measured in 50 μl of 50 m*M* tris-HCL, *p*H 7.5, 5 m*M* dithiothreitol, 100 m*M* KCl, 10 m*M* $MgCl_2$, 10 m*M* $^3$H-labeled dTTP, and 0.1% Triton X-100 containing 2 μg of poly(A), 0.4 μg of $(dT)_{12-18}$ and 20 μl of cell extracts [slightly modified from (*25*)]. For electron microscopic examinations (EM), cells were fixed in 1% glutaraldehyde, processed according to standard methods, and observed under a Phillips EM300. For testing supernatant activity, cell supernatants collected during medium changes were filtered through 0.45-μm (Millipore) filters and used for resuspending pellets of Raji cells or PHA-treated CBL (3 days, 50 μg/ml). Raji cells were tested for the induction of EA 24 hours after supernatant addition. Supernatant-treated and control CBL were incubated in 96-well microtiter plates and tested for [$^3$H]thymidine incorporation into cellular DNA.

| Marker or feature | Positivity or negativity |
|---|---|
| OKT3 | − |
| OKT4 | + |
| OKT8 | − |
| OKT11 | + |
| M-1 | − |
| B-1 | − |
| IgM μ | − |
| Tac | + |
| EBV-R | − |
| EBV-MA | + |
| EBNA's | − |
| EBNA-1 | − |
| EBNA-2 | − |
| EA | + |
| VCA | − |
| ATLA | − |
| RT activity | − |
| Herpesvirus particles | − |
| Retrovirus particles | − |
| EBV genome | + |
| Supernatant infectivity | − |
| Growth stimulation of PHA-treated CBL | − |

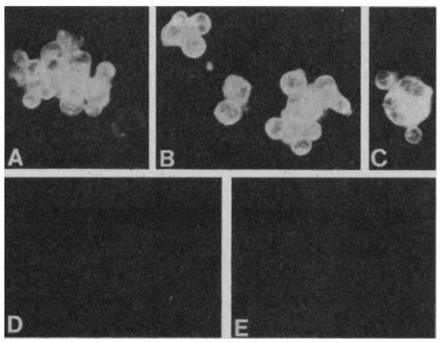


Fig. 2. Detection of EBV-determined early antigens (EA) in HBD-1 cells. Cells were fixed in acetone and stained by indirect immunofluorescence for EA (A, B, C), VCA (D), and EBNA (E) as described in legend to Table 1. (A to E, ×450).

antibody to Tac receptor (*11*), addition of IL-2 to the culture medium was not necessary for the establishment of the lines or their cultivation. Furthermore, HBD-1 cells apparently did not secrete IL-2 since the HBD-1–conditioned medium did not stimulate [$^3$H]thymidine uptake in phytohemagglutinin (PHA)-treated CBL (Table 1). Cytogenetic analysis revealed that cells of the HBD-1 line had a normal karyotype, as expected in cells obtained from healthy donors. Although immortal, HBD-1 cells did not have a fully malignant phenotype, as determined by their inability to grow in soft agar or to induce tumors in nude mice. The cell surface phenotypic profile of HBD-1 cells, and most of the other properties, have remained virtually unchanged during approximately 20 months of continuous culture.

In spite of their T-cell origin, HBD-1 cells contained and actively expressed EBV genome. This was demonstrated in several ways:

1) A high proportion of HBD-1 cells (30%) expressed early antigens (EA) (Fig. 2, A to C) and membrane antigens (MA) (20%) (Table 1). Surprisingly, the cells were negative for EBNA, the only antigens normally expressed in EBV-transformed human B lymphocytes (Fig. 2E and Table 1). The HBD-1 cells were also negative for the EBV capsid antigen (VCA) and did not secrete infectious virus particles (Table 1), suggesting that the EBV genome was present in a latent state or was incapable of replication.

2) EBV DNA was detected in HBD-1 cells by Southern blotting and hybridization with five large overlapping EBV cosmid clones covering approximately 90% of the viral genome (Fig. 3). Analysis of Hirt cell extracts (*12*) revealed a number of virus-specific bands hybridizing to four of the cosmid probes (Fig. 3, A to D). No hybridization could be detected with a cosmid clone spanning the Bam HI-F-O region. The EBV DNA band pattern did not match that of either parent B95-8 or HH514-16, indicating the presence of a rearranged or incomplete viral genome. Similar observations were made by Griffin *et al.* (*13*) after transfection of EBV DNA into monkey epithelial cells. It was not possible, therefore, to determine whether the HBD-1 cells contained DNA from B95-8 or HH514-16 strains, or both. Under the conditions of high stringency used here, the probes did not cross-hybridize with any control lines, which included a number of EBV-negative T- and B-cell lines and normal T lymphocytes.

3) EBV-specific RNA's were identified by Northern blotting. Figure 4 shows messenger RNA's (mRNA's) hybridizing to several cloned DNA probes from divergent regions of the viral genome. The presence of full-size viral RNA transcripts of about 3.2 kb was evident when we used a probe to the Bam HI–W region (Fig. 4A), while an Eco RI C+H probe hybridized to mRNA's of approximately 3 kb, 1.5 kb, and 0.7 kb (Fig. 4C). There was no hybridization to a Bam HI–K probe (Fig. 4B), demonstrating absence of transcripts from the EBNA-1 coding region, while a low copy level of 3.7-kb mRNA was observed in the Raji control line. The RNA transcripts were comparable in size with those obtained from an EBV-positive cell line Raji, whereas an EBV-negative T-cell line 1301 (*14*) gave no hybridization signal.

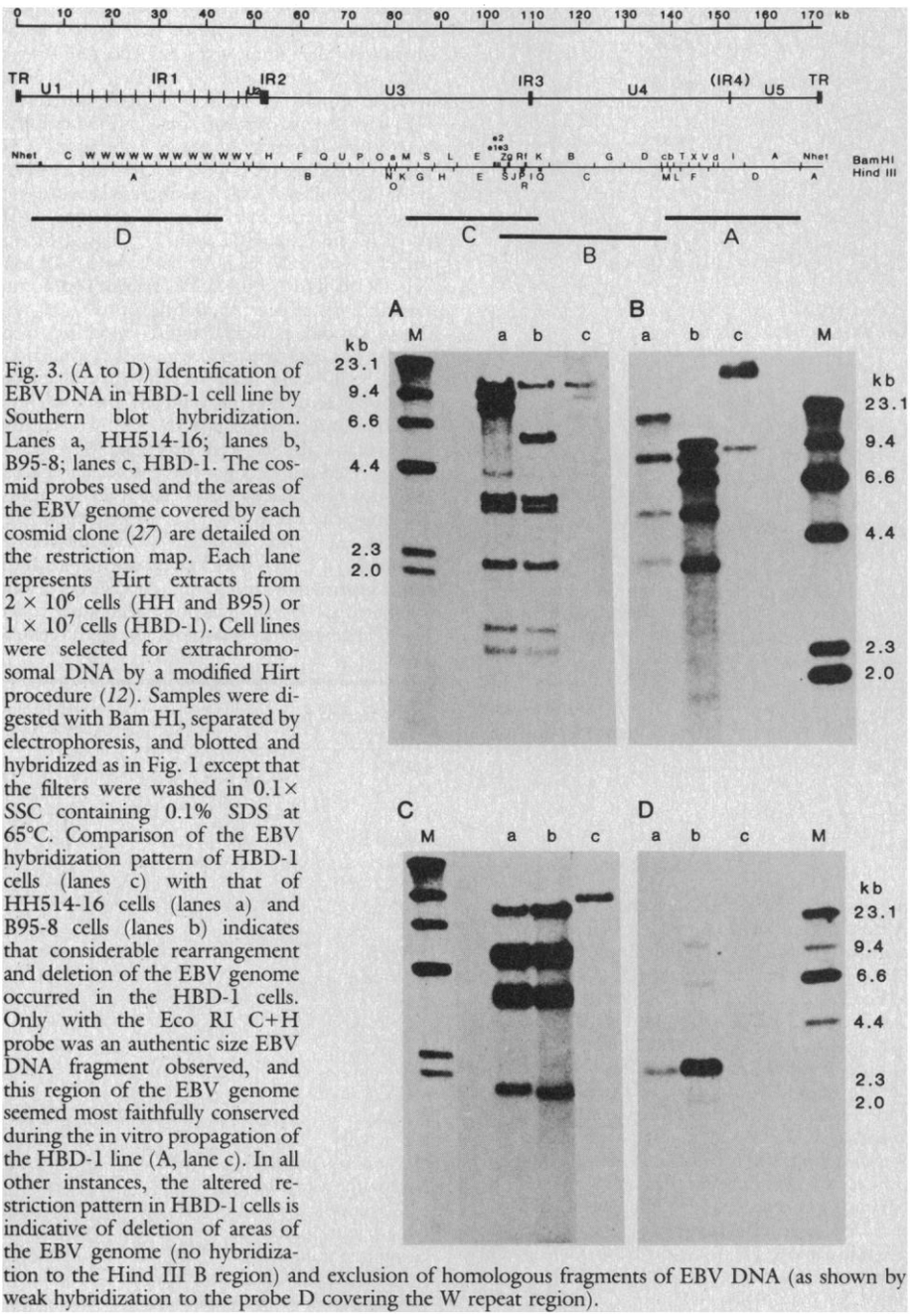


Fig. 3. (A to D) Identification of EBV DNA in HBD-1 cell line by Southern blot hybridization. Lanes a, HH514-16; lanes b, B95-8; lanes c, HBD-1. The cosmid probes used and the areas of the EBV genome covered by each cosmid clone (*27*) are detailed on the restriction map. Each lane represents Hirt extracts from $2 \times 10^6$ cells (HH and B95) or $1 \times 10^7$ cells (HBD-1). Cell lines were selected for extrachromosomal DNA by a modified Hirt procedure (*12*). Samples were digested with Bam HI, separated by electrophoresis, and blotted and hybridized as in Fig. 1 except that the filters were washed in 0.1× SSC containing 0.1% SDS at 65°C. Comparison of the EBV hybridization pattern of HBD-1 cells (lanes c) with that of HH514-16 cells (lanes a) and B95-8 cells (lanes b) indicates that considerable rearrangement and deletion of the EBV genome occurred in the HBD-1 cells. Only with the Eco RI C+H probe was an authentic size EBV DNA fragment observed, and this region of the EBV genome seemed most faithfully conserved during the in vitro propagation of the HBD-1 line (A, lane c). In all other instances, the altered restriction pattern in HBD-1 cells is indicative of deletion of areas of the EBV genome (no hybridization to the Hind III B region) and exclusion of homologous fragments of EBV DNA (as shown by weak hybridization to the probe D covering the W repeat region).

These results demonstrate that we have established a permanently transformed human T-lymphoblastoid cell line that contains actively expressed EBV DNA. The observed rearrangement and deletion of the EBV genome which has occurred in the HBD-1 line is not unexpected. Transfection of cells with large fragments of DNA may result in the deletion of repeat regions of transfected DNA, while DNA recombination could conceivably alter the restriction pattern of stably transformed DNA. We have shown that plasmid DNA when introduced into normal lymphocytes can rapidly be altered as a result of DNA recombination and plasmid instability (*15*). It is not clear at present whether the immortalized state is a direct result of the presence of the EBV DNA. Neither chromosomal aberrations nor endogenous IL-2 seem to play a role in the maintenance of transformation. It is possible that the continuous growth of HBD-1 cells is due to the presence of a transforming human retrovirus supplied, for example, by the normal fibroblasts used as a feeder layer during the first weeks of the culture. However, the cells do not contain any C-type retroviral particles, do not express any measurable reverse transcriptase activity, and do not express HTLV-I–determined antigens [ATLA, see (*16*)]. We thus conclude that HBD-1 cells are negative for animal retroviruses and do not contain HTLV-I, which is the only other human virus known to be capable of transforming T lymphocytes. Another possibility is that the transformation is due to the activation of a cellular oncogene by EBV DNA fragments present in the HBD cells. As shown by Cheah *et al.* (*17*), infection of B lymphocytes or EBV-negative B-lymphocytic cell lines results in up to a 50-fold increase in mRNA of a proto-oncogene c-*fgr*. The HBD-1 cells will provide a suitable system for evaluating this possibility.

One of the most intriguing features of HBD-1 cell lines is the complete lack of expression of EBNA, despite the presence of extrachromosomal EBV DNA fragments. EBNA-1 has been shown to be an essential element for the activity of a *cis*-acting EBV origin of replication in B cells (*18*). However, this role of EBNA-1 in a cell of a non-B lineage has not been investigated. Our data indicate that EBNA-1 may not be essential for plasmid maintenance in T cells. Alternatively, EBNA-1 may be present at an extremely low level and may escape detection with the antiserum that we used in the Western blotting. This would not be the first instance of an active expression of several EBV-determined antigens in the absence of a detectable EBNA. Early antigens, but no other viral proteins, have been expressed

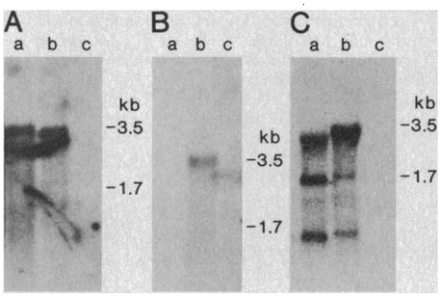


Fig. 4. Northern blot analysis of transcripts from HBD-1 cell line. The probes were: (A) Bam HI–W region; (B) Bam HI–K region; (C) Eco RI–C+H region. Lanes a, poly(A)$^+$ RNA from HBD-1 cell line; lanes b, poly(A)$^+$ RNA from human EBV-positive cell line Raji; lanes c, poly(A)$^+$ RNA from EBV-negative cell line 1301. Each line contained approximately 10 μg of poly(A)$^+$ RNA. In panel B, the apparent signal in lane c is not authentic and represents nonspecific hybridization of the Bam HI–K probe. Glyoxylated ribosomal RNA's served as molecular weight markers. Total RNA was isolated from the cell lines by the guanidium thiocyanate procedure (*26*). Poly(A)$^+$ RNA was selected by two passages over oligo(dT) columns. The RNA's were denatured by glyoxylation, electrophoresed in a 1% agarose gel, and transferred to nitrocellulose essentially as described (*28*). Blots were hybridized with a nick-translated cloned EBV DNA probe at 42°C in 50% formamide, 5× SSC, 3× Denhardt's solution, 0.5% SDS, 50 m*M* $NaPO_4$, 10 m*M* vanadyl ribonucleoside complex, and 100 μg/ml of salmon sperm DNA. Blots were washed at 67°C in 0.1× SSC containing 0.1% SDS.

after microinjection of purified EBV DNA into human diploid fibroblasts (*19*). Infection of EBV receptor–implanted mouse lymphocytes by EBV has resulted in the synthesis of 30 EBV-determined proteins, complete virus replication, but no EBNA expression (*20*). When ectocervix epithelial cells are exposed to EBV, the proportion of EA- and VCA-positive cells is approximately four times higher than that of EBNA-expressing cells (*21*). No EBNA could be detected in monkey epithelial cells transformed with subgenomic fragments of EBV (*13*). We have recently postulated that EBNA may have a role in the down-regulation of the EBV replicative cycle rather than in cell transformation per se (*5, 20*). The results presented here suggest that EBV replication may occur in vivo in cells that are EBNA-negative and, therefore, are not suspected of harboring virus. We now have a cell line from a patient with acute lymphocytic leukemia that has exclusively T-cell surface markers, carries the EBV genome, and expresses EBV-specific polypeptides but is negative for EBNA-1 (*22*). These results raise questions regarding the host cell restriction of EBV. Noteworthy is the fact that only some regions of the EBV genome are present in the HBD-1 line. If these EBV fragments are necessary for maintenance of the immortalized state in HBD-1 cells, more detailed analysis of this EBV DNA may provide information on the regions of the EBV genome that are important for virus-mediated cell immortalization. The HBD-1 cell line and the HBD-1C cell line (a similar T-cell line more recently established by transfection of cord blood lymphocytes with EBV DNA) should provide a useful system for testing this hypothesis.

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# Conodont Survival and Low Iridium Abundances Across the Permian-Triassic Boundary in South China

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**The Permian-Triassic sedimentary sequence of China includes one of the most complete and fossiliferous Paleozoic-Mesozoic boundaries known. Closely spaced sampling across the boundary, which is an important extinction event for most organisms, has produced good conodont faunas that show little diversity change. A drop in conodont abundance is the only apparent response to the extinction event. A low concentration of iridium in the boundary clay (0.002 part per billion ±20 percent), as well as in samples immediately below and above, that range from 0.004 to 0.034 part per billion do not support the proposal of an extraterrestrial impact event at this boundary in China.**

THE PERMIAN/TRIASSIC BOUNDARY (P/Tr) (245 million years ago) has been characterized as the most profound extinction event of the Phanerozoic even though certain organisms may have been relatively unaffected. Recently, the possibility of an extraterrestrial influence for the Late Permian event has been suggested by the reports of iridium anomalies at two localities in China (*1, 2*). This raises the question of how conodonts were able to escape what might have been a major worldwide catastrophe. New conodont and Ir data from an excellent stratigraphic sequence in the Meishan, Changxing area of China, indicate that conodont abundance (but probably not species diversity) was affected at the P/Tr boundary and that the concentration of Ir in the boundary clay is extremely low (0.002 ppb). The environmental factors contributing to the Late Permian extinction event were only effective in reducing conodont abundance and, furthermore, did not involve any increase in Ir abundance.

The discovery of an Ir anomaly at the Cretaceous/Tertiary (K/T) boundary (*3*) with its associated mass extinctions has led to investigations of Ir abundances at other biologic extinction events (*4–11*). It is reported that the Late Permian event resulted in the elimination of approximately 50% of the preservable marine families in the World Ocean, and it is estimated that between 77 and 96% of the marine invertebrate species were affected (*12*). There was a significant decrease in the world terrestrial reptilian faunas at this time as well (*13*). Because the Late Permian extinction "stands alone as the most devastating collapse of the marine ecosystem" (*12*), studies of possible geochemical anomalies, similar to those at the K/T boundary have followed naturally at the P/Tr boundary. An Ir abundance of 8 ppb has been reported for the 4 cm of P/Tr boundary clay in the Meishan area of Changxing (*1*), and an abundance of 2 ppb was reported for the P/Tr boundary in the Shangsi section, Guangyuan County of Sichuan Province (*2*) (Fig. 1). Both occurrences were considered evidence of an extraterrestrial event at the end of the Permian.

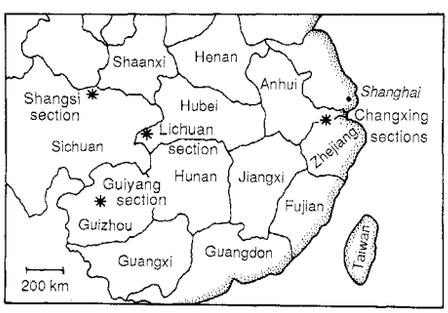


Fig. 1. Localities of P/Tr sections in South China referred to in this report. Data on Changxing sections are from this report and (*1, 5*); on the Lichuan section from this report; on the Shangsi section, from (*2*); and on the Guiyang section, from (*5*), Table 2.

The Changxing report is of particular interest because the section at this locality may represent the best of the few uninterrupted Permian-Triassic marine intervals worldwide. Complete sequences in Kashmir, the Salt Range, South China, central and northern Iran, and the Kap Stosch area of East Greenland have been reported, but recent work indicates that the relation between the Permian and Triassic in at least some of these areas is disconformable (*14*). Because of this, the Changxing section has been recommended as the world stratotype for the Late Permian (*15*) and is of particular importance for documentation of the validity of an extraterrestrial (terminal) Permian event.

A novel aspect of the Late Permian extinction, known for more than a decade, is that Late Permian conodont species studied in Iran and elsewhere range across the P/Tr boundary relatively unaffected (*16*). During the whole of the Late Permian interval there were at least 50 species and eight genera of conodonts although there probably were never more than five to eight species living at one time (*17*). In the Salt Range, Kashmir, and northern and central Iran, five or six conodont species, the total number known in the Late Permian, all range across the P/Tr boundary (*16*). Because five species are within the range of normal Late Permian conodont population levels (*17*) the occurrence of this number of the same species on either side of the P/Tr boundary suggests that the factors responsible for the devastating collapse of the marine ecosystem at this time had little effect on conodont diversity.

The report of an Ir anomaly at the Chinese P/Tr boundary renewed our interest in this extinction event and the survival of conodonts. In order to determine a precise conodont sequence in what may be the best P/Tr section (and from the same sediment layers reported to show the Ir anomaly), detailed sampling for conodonts was undertaken in 1984–85 by C. Y. Wang. In South China, the marine P/Tr boundary occurs in many places, but the most important is the Changxing area of Zhejiang Province (Fig. 1). This section has been studied in detail by a joint Chinese-Japanese team (*15*).

Among the several sections documented in this study is the section in Meishan at Changxing, the stratotype of the Changshing Stage (latest Permian). Section D of

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