One Functional Copy of the Long Terminal Repeat Gene Specifying the Immediate-early Polypeptide IE 110 Suffices for a Productive Infection of Human Foetal Lung Cells by Herpes Simplex Virus

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SUMMARY

The HSV-1/HSV-2 intertypic recombinant Bx1 (28-1) is heterotypic for the repeat sequences flanking the long unique region of the genome (IR_L and TR_L) and expresses both the immediate-early polypeptide IE 110 of HSV-1 and its functionally equivalent polypeptide IE 118 of HSV-2. The genome structures of five subclones of this recombinant and the immediate-early polypeptides they induce have been analysed. Subclone 14 lacked most of the IR_L sequence, including the region from which part of the mRNA for IE 110 is transcribed, and expressed only the HSV-2 IE 118. Subclone 22 lacked almost all of TR_L including the gene for IE 118 and induced only the HSV-1 IE 110. Since both subclones produced viable progeny in HFL cells it follows that expression of only one copy of the equivalent genes in TR_L and IR_L, here coding for the distinguishable polypeptides IE 110 or IE 118, is sufficient for successive complete cycles of virus replication.

INTRODUCTION

Repeated polynucleotide sequences, ranging in complexity from the subgenic to the genomic level, are a common feature of the genetic material of prokaryotes, eukaryotes and their viruses. The genomes of herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) both consist of two segments L (121 kb) and S (24.5 and 27.2 kb) comprising unique sequences U_L and U_S, bounded by inverted repetitions TR_L and IR_L (10.5 kb) and TR_S and IR_S (6.0 kb) respectively (Fig. 3a; Sheldrick & Berthelot, 1974; Hayward et al., 1975b; Wilkie, 1976; Cortini & Wilkie, 1978). A terminal redundancy, known as the a sequence, of about 265 base pairs (bp) for serotype 1 (HSV-1) strain KOS, 327 to 459 bp for HSV-1 strain 17, and 252 bp for serotype 2 (HSV-2) strain HG52 (A. J. Davison & N. M. Wilkie, unpublished results), has been reported and this sequence is repeated in inverted orientation at the joint between the L and S segments (Sheldrick & Berthelot, 1974; Grafström et al., 1974, 1975; Wilkie & Cortini, 1976; Wagner & Summers, 1978; A. J. Davison & N. M. Wilkie, unpublished results). The L and S segments usually invert freely about the joint, resulting in equal amounts of both orientations of each segment in virion DNA (Hayward et al., 1975b; Clements et al., 1976: Delius & Clements, 1976).

Little is known about the functional significance of the genome structure. Gene mapping studies using analysis of intertypic recombinants between HSV-1 and HSV-2, and transcript mapping with in vitro translation, have located immediate-early genes coding for polypeptides IE 110 (HSV-1) or IE 118 (HSV-2) within TR_L/IR_L, and IE 175 (HSV-1) or IE 182 (HSV-2) within TR_S/IR_S (Preston et al., 1978; Watson & Clements, 1978; Morse et al., 1978; Watson...
et al., 1979; Easton & Clements, 1980; Anderson et al., 1980). The HSV genome is therefore diploid for at least two genes.

Preston et al. (1978) described an intertypic recombinant Bx1 (28-1), which is heterotypic for TR_L/IR_L and which expresses both HSV-1 IE 110 and the HSV-2 IE 118. The L region of this recombinant fails to invert normally and virion DNA contains a marked preponderance of one orientation of L. But the question remains whether expression of both the IR_L and TR_L gene copies is obligatory. In this communication we describe viable subclones of Bx1 (28-1) from which either TR_L or IR_L sequences have been deleted, including the entire coding region for IE 118. Thus, the presence of only one intact TR_L/IR_L sequence is necessary for productive infection in vitro.

METHODS

Cells. BHK clone 13 cells (Macpherson & Stoker, 1962) were grown in Eagle's medium supplemented with 10% calf serum. HFL cells are a line of human foetal lung cells established by Dr B. Carritt in the Institute of Genetics, Glasgow. They were grown in Eagle's medium supplemented with 10% foetal calf serum and 1% non-essential amino acids (Flow Laboratories).

Virus. The wild-type strains of HSV-1 (17) and HSV-2 (HG52) and mutants ts B (derived from strain 17) and ts 1 (derived from strain HG52) have been described previously (Brown et al., 1973; Timbury, 1971). The isolation and properties of recombinants Bx1 (28-1) and Bx1 (31-2) have also been described (Preston et al., 1978). Subclones of Bx1 (28-1) were picked from under agarose at 31 °C and purified by three cycles of plaque purification (Wilkie et al., 1978). Of 20 subclones analysed, 17 had further crossovers in the inverted repeated regions, two were deleted in TR_L or IR_L, and only one had the parental structure (A. J. Davison & N. M. Wilkie, unpublished results).

Chemicals. Cycloheximide and actinomycin D were purchased from Sigma. All other chemicals were purchased from either BDH or Bio-Rad.

Isotopic labelling of virion DNA. Virus DNA was labelled in vivo with 32P as described by Preston et al. (1978) and DNA isolated from virions by phenol extraction (Wilkie, 1973). For preparation of in vitro labelled DNA, virus DNA was prepared by phenol extraction and rate-zonal centrifugation (Wilkie, 1973; Wilkie & Cortini, 1976) and subsequently labelled with α-deoxyribonucleoside [32P]triphosphates (The Radiochemical Centre, Amersham) by nick translation (Rigby et al., 1977). After restriction endonuclease digestion, fragments were excised from agarose gels and purified by hydroxylapatite chromatography (Wilkie, 1976), and then dialysed.

Restriction endonuclease analysis. EcoRI was obtained from MRE, Porton Down, Wiltshire, U.K. and other restriction endonucleases from Bethesda Research Laboratories or New England Biolabs. Complete digestion of labelled HSV DNA was carried out in the presence of 0-2 μg lambda DNA (Bethesda Research Laboratories) in 50 μl 6 mM-tris–HCl pH 7-5, 6 mM-MgCl2, 6 mM-2-mercaptoethanol, 20 mM-KCl, 0-1% (w/v) bovine serum albumin. Gel electrophoresis was carried out using agarose concentrations varying between 0-4 and 1-0% (Wilkie & Cortini, 1976) and polyacrylamide gel electrophoresis (PAGE) of small DNA fragments was done by the method of Maniatis et al. (1975). Autoradiographs of dried gels were made with Kodak X-Omat H film.

Blot hybridization. DNA fragments of restriction digests were transferred to nitrocellulose sheets (BA80; Schleicher & Schüll) by the method of Southern (1975). Blot strips were then pre-incubated overnight at 37 °C in 60% formamide, 3 × SSC (0-045 mM-trisodium citrate, 0-45 mM-NaCl), Denhardt's solution (0-02% polyvinyl pyrrolidone, 0-02% Ficoll 400, 0-02% bovine serum albumin), 100 mg/ml denatured salmon sperm DNA, and 0-1% SDS. The strips were hybridized under the same conditions with denatured nick-translated 32P-labelled DNA, washed at 60 °C in 2 × SSC, and autoradiographed.
Radiosotopic labelling of IE polypeptides. Immediate-early polypeptides were induced in HFL cells using the protocol described by Preston et al. (1978). Both BHK and HFL cells were able to support multiple rounds of infection with the viruses used. Briefly, cells were infected at 37 °C and incubated for 5 h in the presence of cycloheximide. The drug was removed and polypeptides labelled with [35S]methionine in the presence of actinomycin D.

To harvest samples, the labelling medium was first removed. For PAGE analysis of polypeptides from whole cells, the cells were lysed and the proteins denatured directly on the plastic surface on which the cells were growing (Marsden et al., 1978). For PAGE analysis of nuclear polypeptides, nuclei were first separated by the following procedure: cells were scraped from the dish into ice-cold PBS, pelleted by centrifugation at 2000 rev/min for 5 min, resuspended in 1 ml ice-cold isotonic buffer containing 0.65% NP40 and vortexed briefly. After 10 min incubation the nuclei were pelleted by centrifugation at 2000 rev/min for 5 min and the supernatant discarded. The nuclei were resuspended and boiled in denaturing buffer (Marsden et al., 1976).

SDS gel electrophoresis. Samples were separated by electrophoresis on 5 to 12% gradient gels or 7.5% gels cross-linked with N,N'-methylenebisacrylamide, and autoradiographs prepared as described previously (Marsden et al., 1978).

Polypeptide nomenclature. Polypeptides were labelled according to the estimated mol. wt. on gradient gels (Marsden et al., 1976): the nomenclature adopted has been defined (Marsden et al., 1978).

RESULTS

Recombinant genome structures

Independent plaques of Bx1 (28-1) were isolated by three cycles of plaque purification at 31 °C, and stocks were prepared. Virion DNA was 32P-labelled in vivo and restriction profiles were compared on agarose gels, using restriction endonucleases Xbal, HindIII, EcoRI, BglII, HpaI, KpnI and BamHI. Deduction of the genome structure of Bx1 (28-1) has been dealt with in detail (Preston et al., 1978), and the same analytical principles were applied to the subclones. We present the more pertinent results in Fig. 1 and 2 with relevant restriction maps in Fig. 3, and the deduced genome structures focusing on the repeat regions in Fig. 4. Those endonucleases which do not cleave both pairs of repetitions generate half- and quarter-molar bands in restriction profiles (Hayward et al., 1975b; Clements et al., 1976; Wilkie & Cortini, 1976). These bands represent restriction fragments mapping at the termini and the joint between L and S, and result from the presence of equal amounts of the four genome isomers within a DNA population. Subclones 11, 29 and 22 were normal in this respect but subclones 1 and 14, like the parent Bx1 (28-1), showed anomalous molarities of terminal and joint fragments, for example predominance of HSV-2 HpaI g over HSV-1 HpaI m in subclone 1, demonstrating that they possess virion DNA with the L segment largely in one orientation. In principle, the subclone structures shown in Fig. 4 can be derived by recombination, plus in two cases deletions, involving only the parent Bx1 (28-1).

Restriction profiles of the DNA of subclones 14 and 22 suggested the absence of sequences mapping in IR_1 and TR_1 respectively. For example, Fig. 1 shows that subclone 14 lacked HSV-1 HpaI m and subclone 22 lacked HSV-2 HpaI g. A novel EcoRI fragment from the L terminus migrated below HSV-2 EcoRI l in each case; the fragment from subclone 14 was in low abundance because the L segment was present largely in the orientation depicted in Fig. 4. The subclone 22 fragment which migrated in the position of HSV-2 EcoRI f is explained as the joint fragment consisting of the novel L terminal fragment covalently linked to HSV-2 EcoRI m. A mixture of nick-translated HSV-1 and HSV-2 BamHI joint fragments was hybridized to nitrocellulose filters containing KpnI and BamHI fragments of the DNA of subclones 14 and 22, in order to identify joint and terminal fragments, several of which
Fig. 1. Autoradiographs of restriction digests of \textit{in vivo} $^{32}$P-labelled virion DNA of Bx1 (28-1) subclones for (a) $EcoRI$ (0.4 % agarose), (b) $HpaI$ (0.4 %), (c) $KpnI$ (0.5 %), (d) $BamHI$ (1 %). Parental HSV-1 \textit{ts} B (T1) and HSV-1 \textit{ts} 1 (T2), Bx1 (28-1) (R) and subclones (11, 14, 22) are shown. Letters refer to specific HSV-1 or HSV-2 DNA fragments, the physical map locations of which are given in Fig. 3.
Fig. 2. Autoradiographs of restriction digests of in vivo $^{32}$P-labelled virion DNA of Bx1 (28-1) subclones for (a) EcoRI (0.1 %) agarose, (b) HpaI (0.4 %), (c) KpnI (0.5 %), (d) BamHI (1 %). Parental HSV-1 ts B (t1) and HSV-2 ts 1 (T2) Bx1 (28-1) (R) and subclones (1, 29) are shown. Letters refer to specific HSV-1 or HSV-2 DNA fragments, the physical map positions of which are given in Fig. 3.
co-migrated with, and were therefore obscured by, other bands in the profiles shown in Fig. 1. The results and their interpretation are shown in Fig. 5 (a). Novel fragments from the L terminus had only an a sequence in common with the probe DNA and therefore did not
Expression of HSV long repetitions

Fig. 4. Genome structures of subclones of BxI (28-1). U_L and U_S have been shortened to emphasize details of the repetitive regions, and the a sequences are shown on a larger scale than repetitive regions. Restriction sites within repetitive sequences (B = BamHI, K = KpnI, H = HpaI, R = EcoRI) are shown above the genome for HSV-1 and below for HSV-2. Black denotes HSV-2 DNA and white HSV-1 DNA, cross-hatching indicates an uncertainty in crossover position greater than 200 bp, and crosses signify deleted sequences. The indicated crossover position in U_L was between 0.490 and 0.515 fractional genome units for each subclone. The positions and direction of transcription of the genes coding for IE 110 and IE 118 are shown by solid arrows at the top of the figure (F. J. Rixon & J. B. Clements, personal communication).

Hybridize strongly, allowing them to be distinguished from other joint and terminal fragments. Other faint bands are characteristic of the presence of additional a sequences at the joints and L termini. Comparison of Fig. 1 and 5 (a) shows that HSV-1 KpnI r of subclone 22 was slightly smaller than that of HSV-1 ts B and co-migrated with HSV-2 KpnI no. Faint bands in the KpnI profiles in Fig. 1 are due to slight under-digestion of the DNA samples. No contaminant genome structures were detected in subclones 14 and 22 in these experiments.

Fine structure restriction maps and nucleotide sequences for the a sequences of HSV-1 and HSV-2 have been determined (Wagner & Summers, 1978; A. J. Davison & N. M. Wilkie, unpublished results), and an important feature of the HSV-1 a sequence is that it exhibits size variability, whereas the HSV-2 a sequence does not. SmaI restriction maps of HSV-1 BamHI k (Fig. 3 b) and HSV-2 BamHI g (not shown) have been used to examine the types of a sequences present at the joints and termini of BxI (28-1) subclones (A. J. Davison & N. M.
Fig. 5. (a) Autoradiography of nitrocellulose blot strips containing KpnI or BamHI restriction fragments of subclone 14 (S14) or 22 (S22) DNA, to which nick-translated DNA probes had been hybridized. The probes were: HSV-1 DNA (H), and a mixture of plasmids containing HSV-1 BamHI k and HSV-2 BamHI g (J) (A. J. Davison & N. M. Wilkie, unpublished results). HSV-1 DNA hybridized strongly to HSV-1 fragments in the recombinant genomes, and to a lesser extent to HSV-2 fragments. An explanation of the bands to which the J probe hybridized is given (NLT = novel L terminus; H1 = HSV-1 fragment; H2 = HSV-2 fragment; S = S segment, since the subclone genomes had no KpnI sites in S). (b) Autoradiographs of Smal restriction digests of fragments isolated from nick-translated DNA from Bx1 (28-1) subclones deleted in long repetitive regions. (1) ts B BamHI k; (2) ts B HpaI m; (3) subclone 14 BamHI/HpaI joint fragment; (4) subclone 22 HpaI m cleaved with BamHI as well as Smal; (5) subclone 22 terminal HpaI fragment from TRy.

Wilkie, unpublished results), and some of these data are included in Fig. 4. Fig. 5 (b) shows results from which the nature of a sequences adjacent to the deleted regions in subclones 14 and 22 may be determined. Smal G is a cleavage product of HSV-1 joints and termini and therefore maps completely within the a sequence. It is variable in size and hence the HpaI/BamHI joint fragment of subclone 14 produced a slightly smaller Smal G fragment than the joint or L terminal fragments of HSV-1 ts B (Fig. 5b, tracks 1 to 3). Smal fragments common to the joint regions of HSV-1 and subclone 14 included A, B, E, F, G, M and O, and other fragments from the subclone 22 HpaI/BamHI joint fragment were common to HSV-1 HpaI m but not BamHI s. In contrast, the major BamHI joint fragment of subclone 1 contained Smal sites in identical positions to those of HSV-1 BamHI k, and subclone 29 gave results consistent with the structure shown in Fig. 4 (data not shown). These results are consistent with the deletion of long repetitive sequences and the presence of most or all of an HSV-1 a sequence at the joint of subclone 14. Both L termini of subclone 22 produced Smal G, also smaller than that of HSV-1 ts B (Fig. 5b, tracks 4 to 5). Two of the other three fragments from the novel HpaI terminus (track 5) co-migrated with Smal fragments of HSV-2 HpaI g which were not common to HSV-2 BamHI v, and the third fragment was interpreted as spanning the deleted region (data not shown). In contrast, subclones 1 and 29 possessed normal HSV-2 BamHI v fragments as demonstrated by Smal cleavage (data not
Expression of HSV long repetitions

Table 1. Immediate-early polypeptides induced by HSV

<table>
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<th>HSV-1 IE polypeptide</th>
<th>HSV-2 equivalent IE polypeptide</th>
<th>Probable equivalent ICP*</th>
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<td>6</td>
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<td>†</td>
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<tr>
<td>12</td>
<td>12.5</td>
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† →, No equivalent band detected.
‡ ?, Not known.
§ ND, No data.

shown). These results are consistent with the presence of most or all of the HSV-1 a sequence adjacent to the deleted region in subclone 22. The deleted region of subclone 14 extends over 4.5 kbp and that of subclone 22 extends over 8 kbp.

Immediate-early polypeptides induced in HFL cells

Table 1 lists those IE polypeptides described previously (Honess & Roizman, 1974; Preston et al., 1978; Watson et al., 1979; Easton & Clements, 1980; Fenwick et al., 1980). Table 1 also lists the most probable equivalent polypeptide in the nomenclature of Honess & Roizman (1974). Fig. 6, which is a composite of SDS-polyacrylamide gels from several experiments, shows immediate-early polypeptides induced by HSV-1 (strain 17), HSV-2 (strain HG52) and several intertypic recombinants. In early experiments whole infected cells were analysed by SDS–PAGE (lanes 1 to 10) though in later ones, nuclear extracts were analysed (lanes 11 to 21) since it had been shown that IE 110 partitioned with the nucleus (Pereira et al., 1977). Six HSV-1 coded [175, 136'(143), 110, 87, 68, 63] and six HSV-2 coded (182, 138, 134–132, 118, 67, 64) IE polypeptides can be identified in these experiments.

As observed previously (Preston et al., 1978), recombinants Bx1 (31-2) and Bx1 (28-1), which are heterotypic for the repeats bounding UL, induced both IE 110 (HSV-1) and the equivalent HSV-2 polypeptide IE 118. Fig. 6 shows that subclones 1 and 29, which are heterotypic for IRL and TRUL, induced both IE 110 and IE 118. The latter inverts normally in L and S but subclone 1 has virion DNA with the L segment predominantly in one orientation, and therefore whether or not the L segment inverts normally seems not to affect expression of the IE 110/118 genes. Subclone 14 which lacks HSV-1 sequence from IRUL (including a region from which the 5' end of the mRNA for IE 110 is transcribed; F. Rixon & B. Clements, personal communication), induced only the HSV-2 IE 118. Conversely, subclone 22, which lacks HSV-2 sequence from TRUL (including the whole of the gene for IE 118; A. Easton & B. Clements, personal communication) induced only the HSV-1 IE 110. Subclone 11 induced IE 118 and a polypeptide which migrated faster than IE 110. Fig. 4 shows that a crossover exists within the DNA sequences coding for the HSV-1 IE 110 mRNA. We conclude that the altered polypeptide arose as a consequence of the crossover position in IRUL. The apparently smaller size may result from non-co-linearity of initiation or termination codons for the two polypeptides, a deletion of some coding sequence as a result of the crossover, or of altered processing of the hybrid polypeptide.
Fig. 6. Autoradiograph of IE polypeptides induced in HFL cells by HSV-1 ts B (T1), HSV-2 ts 1 (T2), recombinants Bxl (31-2) and Bxl (28-1) and subclones (S) derived from Bxl (28-1). Also included are mock-infected (MI) samples. IE polypeptides from whole infected cells were separated on 5 to 12% gradient gels (lanes 1 to 10) or nuclear extracts were made and separated on 7.5% gels (lanes 11 to 21). Numbers to the left of lane 1 and to the right of lane 21 show the apparent mol. wt. (× 10^-3) of HSV-2 IE polypeptides. II, HSV-1 polypeptide; O, HSV-2 polypeptide; +, altered polypeptide. The symbols are placed to the left of the track to which they refer.
DISCUSSION

The results presented here show that deletions of sequences from either TR$_L$ or IR$_L$ are produced under normal culture conditions and that the resultant virus is viable in the in vitro tissue culture system used. The deleted regions contain sequences coding for IE 110/118, demonstrating that expression of only one of the two copies of this gene which are normally present is sufficient for a productive infection. Even extensive deletion of TR$_L$ results in viable virus, as evidenced by subclone 22 which lacks a considerable sequence beyond the gene coding for IE 118. Clearly, any other genetic information in the long repetitions is not required in diploid amounts for continued virus replication in tissue culture. One other protein has been mapped in TR$_L$/IR$_L$ (Morse et al., 1978) but for reasons outlined elsewhere (Watson et al., 1979) we cannot identify the equivalent polypeptide in our nomenclature. Another recombinant, RE4, has a deletion which extends from the joint between L and S, through the entire IR$_L$ region and at least 0.3 kbp into U$_L$ (A. J. Davison & N. M. Wilkie, unpublished results). Nonetheless, fine structure mapping shows that the a sequence is retained at the L terminus and at the joint between L and S in every deleted genome we have analysed. This suggests that a sequences at the terminus and joint may be necessary for non-defective virus genome replication, or are regenerated during DNA replication, or that retention of a sequences is a consequence of the mechanism leading to these deletions which is presently not known. Extensive deletions of the type described for the long repetitions have not been observed in the short repetitive regions of intertypic recombinants. This difference could be due to a requirement for, or at any rate a considerable selective advantage of, functional diplody of short repetitive sequences for productive infection, or could be a result of the mechanism of deletion.

Our observations raise the question why all field isolates examined to date are diploid for repetitive sequences (Hayward et al., 1975 a; Skare et al., 1975; Lonsdale et al., 1979). We can envisage several explanations. The failure to observe naturally occurring large deletions in repetitive regions may be a consequence of the low frequency with which such deletion events occur in nature. The deleted genomes described have been isolated from an intertypic recombinants with homotypic diploid regions (Preston et al., 1978; Marsden et al., 1978; deleted in TR$_L$ or IR$_L$. No equivalent deletions have been observed in over 100 intertypic recombinants with homotypic diploid regions, (Preston et al., 1978; Marsden et al., 1978; Chartrand et al., 1979, 1980; A. J. Davison & N. M. Wilkie, unpublished observations), thus the frequency at which deletions occur or are isolated must be lower. No field isolate has been reported to possess a recombinant structure with heterotypic diploid regions; thus, if deletions are naturally being generated, their frequency must be so low as to have escaped isolation in the relatively small number of field isolates examined to date. The reasons why heterotypic recombinants with deletions in the long repetitive regions were so readily isolated is not known, nor is it known whether this is primarily a result of the frequent generation of deletions or of their subsequent selective advantage. It should be noted that deleted TR$_L$ or IR$_L$ sequences theoretically can be regenerated in homotypic genomes by recombination, whereas this is not possible in heterotypic recombinants.

If viruses with deletions in the genome were at a selective disadvantage for growth, naturally occurring deletion mutants would not survive. We have not yet compared the detailed growth characteristics of deletion mutants with wild-type strains in vitro or in vivo. It is possible that the increased gene dosage resulting from diploid sequences confers an advantage in establishing an infection at low multiplicities, or in cell types naturally resistant to virus infection or replication, and this might be particularly important in the natural host.

It is also possible that the presence of the inverted diploid regions, or the expression of genes contained in them, is essential for an unidentified function of the virus. The HSV genome bears some structural resemblances to transposable elements but it remains to be seen
whether this is functional in any of the contexts in which the genome interacts with host cells, including latency and cellular transformation. In our opinion an unlikely alternative is that the presence of diploid sequences is of no crucial consequence to herpes simplex virus, but merely results from a propensity of DNA to replicate wherever possible. Nevertheless, with the data now available it should soon be possible to replace diploid sequences with other genes and, by examining the regulatory consequences, determine the effect of ploidy upon virus function.

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REFERENCES


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