RAPID COMMUNICATION Jpn. J. Cancer Res. 80, 612–616, July 1989

Rearranged Epstein-Barr Virus Genomes and Clonal Origin in Nasopharyngeal Carcinoma

Misuzu Shimakage,[1,4] Masashi Chatani,[2] Nobuko Ikegami[1] and Kanji Hirai[3]

[1]*Clinical Research Institute, Osaka National Hospital, 2-1-14 Hohenzaka, Chuo-ku, Osaka 540,* [2]*Department of Radiation Therapy, The Center for Adult Diseases, Osaka, Higashinari-ku, Osaka 537 and* [3]*Department of Virology and Immunology, Medical Research Institute, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113*

Epstein-Barr virus (EBV) is known to be associated with two malignant diseases, nasopharyngeal carcinoma (NPC) and endemic Burkitt's lymphoma. In this study, the genomes of EBV in biopsy specimens from 4 NPC patients in Japan were analyzed using Southern blot hybridization. The NPC tissues of all examined cases contained rearranged EBV genomes whose *Bam*HI H fragments were larger than those of prototype EBV genomes. One of them had a *Bam*HI fragment containing contiguous sequences of *Bam*HI Y and H. A single-sized EBV DNA terminus was observed in these NPC tissues, implying the evolution of the carcinoma from a single EBV-infected cell.

Key words: Epstein-Barr virus — Nasopharyngeal carcinoma — Southern blot hybridization — EBV nuclear antigen

Nasopharyngeal carcinoma (NPC) is the most frequent cancer in adult males in southern China. However, NPC patients are less common in Japan, and their clinical symptoms are somewhat different from those of Chinese patients with NPC. For example, the size of the tumor in Japanese is much smaller than that of Chinese. The detection of Epstein-Barr virus (EBV) genomes in NPC tissues has been reported by several workers.[1–3] Raab-Traub and Flynn described clonal proliferation of NPC based on detection of the terminal structure of EBV DNA.[4] However, very little is known about the precise role of EBV in the pathogenesis of NPC. In this communication, we report the detection in NPC tissues of rearranged EBV DNA which is of monoclonal origin.

Four male patients clinically diagnosed as NPC had histopathologically non-keratinized squamous cell carcinoma (patients Izu and Tan) and undifferentiated carcinoma (patients Tak and Har). These patients were admitted to the Center for Adult Diseases, Osaka, in 1988. Their clinical diagnosis and titers of antibody to EBV are summarized in Table I. The histopathology of these tumors was evaluated according to the WHO classification. TNM classification and stage grouping of NPC were based on the UICC classification (1978). In addition to NPC, 2 cases (Mur and Kon) of non-epithelial malignant tumors of the nasopharynx were included for the comparison with NPC. The four NPC patients were treated with 60–70 Gy of X-ray irradiation and all the tumors disappeared, indicating that there was no marked difference in X-ray sensitivity among these patients. Antibody titers to viral capsid antigen (VCA) and early antigen (EA) of EBV was determined using commercial slides (Wako Pure Chemicals, Osaka) by an indirect immunofluorescence method. High titers of IgA antibody specific to VCA and IgG antibody specific to diffuse and restricted EA were detected in sera from the NPC patients, whereas the titers of these antibodies were low in sera from the patients with non-epithelial malignant diseases. Since antibody responses to EBV nuclear antigen(EBNA)1 and EBNA2 were reported to differ in cases of chronic active EBV,[5] antibody titers to these antigens were examined using cell lines expressing EBNA1 or EBNA2 by a streptavidin-biotin indirect immunofluorescence method. These cell lines were established in our laboratory by transfecting NIH 3T3 cells with recombinant pZip-Neo-SV(X)1/EBV DNA plasmids containing the coding region for EBNA1 or EBNA2 (obtained from E. Kieff, Harvard University).[6] Antibody titers to EBNA1 and EBNA2 were detected in sera from all four NPC patients and the two patients with non-epithelial malignant diseases. However, anti-EBNA1/anti-EBNA2 ratios did not appear to be related to the clinical symptoms of NPC.

To examine the presence of EBV DNA in these tumor tissues, DNAs extracted from NPC tissue specimens and EBV genome-positive Raji and B95-8 cells were subjected to Southern blot hybridization as described previously.[7] The EBV DNA probes used were derived mostly from our pBR322-EBV (B95-8 strain) DNA recombinant library (Hirai, unpublished). The locations of these frag-

[4] To whom correspondence should be addressed.

Table I. Clinical Diagnosis and Antibody Titers to EBV Antigen in NPC Patients

Patient (age)	Tumor[a]	Histology[b]				Antibody titer					
						VCA			EA	EBNA1	EBNA2
		T	N	M	S	IgA	IgG	IgM	IgG	IgG	IgG
NPC											
Izu (17)	NSC	4	3	0	4	160	160	<10	320	640	640
Tak (23)	UC	3	3	0	4	320	320	<10	320	1280	80
Tan (79)	NSC	2	1	0	3	160	1280	<10	160	1280	80
Har (40)	UC	4	0	0	4	80	320	<10	640	320	160
Control											
Mur (38)	ML	—	—	—	1	10	160	<10	10	80	640
Kon (35)	PC	—	—	—	—	<10	160	<10	<10	320	160

a) NSC, non-keratinized squamous cell carcinoma; UC, undifferentiated carcinoma; ML, malignant lymphoma; PC, plasmacytoma.
b) TNM classification (T, N, M) and stage grouping (S) according to the UICC classification.

ments on the EBV genome are indicated in Fig. la. The recombinant plasmids pM-BamH2 and pJ-HKA7 used for the determination of EBV type A and type B, respectively, were donated by G. W. Bornkamm.[8]

EBV DNAs were detected in all four NPC specimens (Fig. 1b) but not in non-epithelial tumors (data not shown) by Southern blot hybridization with ^{32}P-labeled *Bam*HI C, E, H and M fragments. Of these fragments, the *Bam*HI E and H fragments in NPC EBV DNA appeared to be smaller and larger, respectively, than those in Raji EBV DNA. Since the *Bam*HI E in EBV producer B95-8 cells was reported to contain additional DNA sequences which were deleted in latent Raji EBV DNA,[9] the smaller size of *Bam*HI E in NPC EBV DNA may account for the replication defect in these latent viral DNAs. To confirm the sizes of *Bam*HI H fragments in these NPC EBV DNAs, the labeled probe DNA was removed from the same blotted filter and then re-hybridized with EBV A type specific pM-BamH2 DNA containing the carboxyl-terminal portion of EBNA2 gene[8] located on the *Bam*HI H of EBV DNA (Fig. la and c). The bands identified as a *Bam*HI H fragment in Fig. 1c are marked with a closed circle in Fig. 1b. The sizes of *Bam*HI H fragments for Izu, Tan, Har and Raji were 6.1, 8.0, 7.0 and 5.9 kilobases (kb), respectively. The *Bam*HI H of Tak EBV DNA was not well detected with the probe DNA, probably due to the small amount of EBV DNA. However, the size of *Bam*HI H of Tak EBV DNA was estimated to be 6.1 kb from the faint band marked with an open circle in Fig. 1b. In contrast, these *Bam*HI H bands of NPC EBV DNA were not detected with the pJ-HKA7 DNA probe specific to EBV type B (data not shown). These results also indicate that NPC tumor tissues from at least three patients (Izu, Tan and Har) contain type A specific EBV DNA. As shown in Fig. 1d, we also detected the *Bam*HI W and Y sequences of EBV DNA in these NPC tumor tissues. Although the *Bam*HI W and Y fragments of Tak EBV DNA may not be seen in Fig. 1d, these fragments with the same size as the EBV DNAs in NPC tissues from Izu and Har and prototype EBV DNAs in B95-8 and Raji cells were faintly detectable in the original X-ray film. It is noteworthy that these probes hybridized to *Bam*HI W and a fragment of 8.0 kb in *Bam*HI digests of Tan EBV DNA. Since the 8.0 kb fragment can be hybridized with a pM-BamH2 probe (Fig. 2c) and *Bam*HI Y (data not shown), it is probably a fused fragment of *Bam*HI H (5.9 kb) to *Bam*HI Y (1.8 kb) of a prototype EBV (B95-8) DNA. During the preparation of this paper, we learned that Fahraeus *et al.* had also found the 8.0 kb fused fragment in *Bam*HI digests of EBV DNA from three cases of Chinese NPC.[10]

To investigate the clonality of NPC malignancy, the structure of the fused EBV DNA termini in these NPC tumor tissues was examined using Southern blot hybridization with ^{32}P-labeled terminal fragment of EBV DNA (Fig. 2). The probe DNA containing the terminal repeats (Fig. 2a) was created by subcloning a 7 kb *Eco*RI-*Bam*HI fragment from the fused termini *Eco*RI D-I, Jhet (donated by E. Kieff)[11] into the *Bam*HI and *Eco*RI sites of the plasmid vector pSV2-neo. This probe DNA hybridized to the heterogenously sized *Bam*HI Jhet and Hhet of B95-8 EBV DNA, and to a single band, *Bam*HI A end, representing the fused termini of the circular plasmid EBV DNA in Raji cells (Fig. 2b and c), as reported by Raab-Traub and Flynn.[4] Only one fused terminal fragment was detected with the probe in *Bam*HI digests of EBV DNA from all four NPC tissues (Fig. 2 c). However, the sizes of these fragments varied among these tumor specimens, because of the variable number of

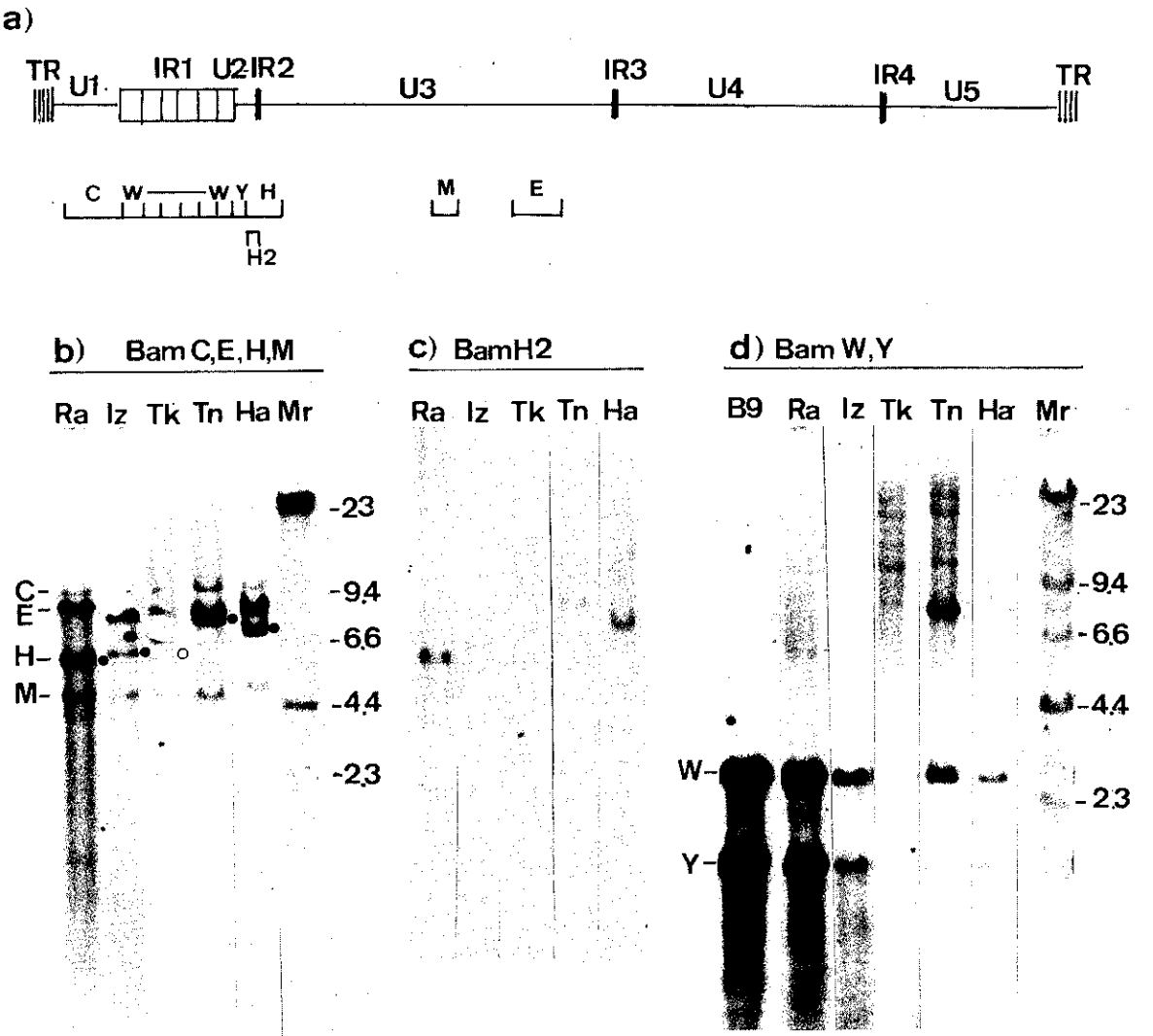

Fig. 1. Detection of EBV DNA in NPC biopsies. (a) The physical structure of the EBV genome is indicated in the top line. The EBV genome is divided into five domains with unique sequences (U1–5) bounded by direct tandem repeats located at the termini (TR) and at internal sites (IR1–4). The locations of EBV *Bam*HI fragments used as hybridization probes are shown below. (b–d) The *Bam*HI digests of total DNAs from NPC tissues (Iz, Izu; Tk, Tak; Tn, Tan; Ha, Har), Raji cells (Ra) and B95-8 cells (B9) were subjected to Southern blot hybridization with (b) *Bam*HI C, E, H and M, (c) pMBamH2 and (d) *Bam*HI W and Y. Closed and open circles in (b) indicate the *Bam*HI H-derived bands and closed circles represent the bands identified by hybridization in (c). Mr in (b) and (d) represents the DNA molecular weight marker I (Nippon Gene Co., Ltd., Toyama). The sizes of marker DNA fragments are indicated in kb on the right sides of the panels in this figure.

terminal repeats of the plasmid DNA in each specimen. This result indicates that each NPC tissue has one form of the joint termini of the EBV genome, representing a monoclonal origin of these NPC in Japan.

Thus, the NPC tissues in all cases examined contained rearranged EBV genomes in comparison with the prototype EBV DNA. The rearrangement could be classified into two groups, A (Tan and Har) and B (Izu and Tak), because the size of the *Bam*HI H derived fragment from group A is larger than that from group B (Fig. 1c). In addition, group A patients showed somewhat different clinical characteristics from group B patients. Patient Tan had double cancers (NPC and thyroid adenocarcinoma) while patient Har had a flat tumor with diffuse invasiveness. In contrast, the group B patients had a typical small-sized NPC tumor. These results suggest a

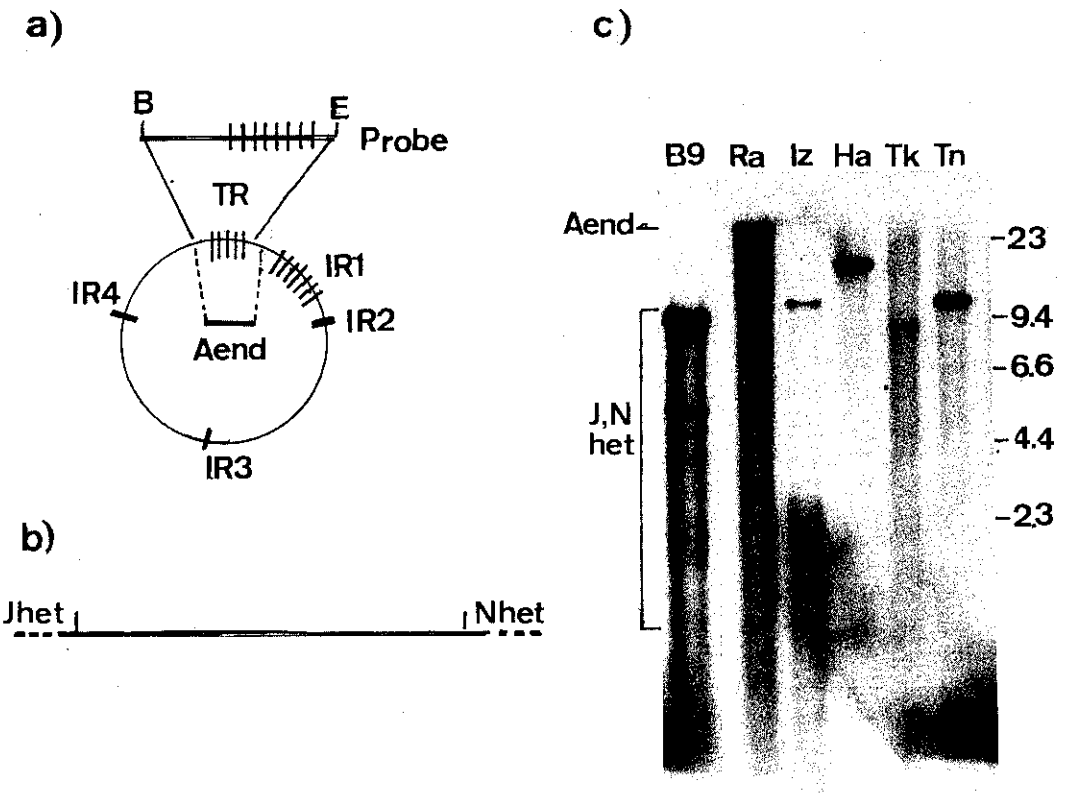

Fig. 2. Analysis of EBV DNA termini in NPC biopsies. (a) Circular form of the EBV genome and the fused terminal *Bam*HI A end detected with a terminal 7.0 kb *Bam*HI (B)-*Eco*RI (E) fragment. Abbreviations for repeats are as indicated in Fig. 1. (b) Linear form of B95-8 EBV DNA and the terminal *Bam*HI J, Nhet fragments. (c) Southern blot hybridization of NPC EBV DNA with ^{32}P-terminal fragment of EBV DNA. Abbreviations for the *Bam*HI digests of total DNA from NPC biopsies and the size markers are as indicated in Fig. 1.

tight association of EBV with NPC in Japan. However, more NPC specimens must be examined for the structure of EBV DNA to determine whether the rearranged EBV DNA is associated with specific clinical characteristics of NPC.

In addition to a study on the monoclonal origin of NPC,[4] the clonal pathogenesis of EBV-related diseases has been investigated in several laboratories, and findings include the oligoclonal or monoclonal origin of B-cell lymphoma,[12, 13] polyclonal B-cell lymphoproliferation of infectious mononucleosis[14] and monoclonal T-cell lymphoproliferation.[15] It is noteworthy that cell populations involved in the acute phase of virus infection are commonly polyclonal, while malignant proliferative cells resulting from viral infections are monoclonal. We previously reported the change in the titers of serum antibody specific to EBNA in NPC.[16] Clinical follow-up study on these patients suggests a good prognosis. However, further follow-up studies are planned to examine serum antibody levels specific to each EBV antigen, such as EBNA1 and EBNA2, in NPC patients.

This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

(Received February 20, 1989/Accepted May 6, 1989)

REFERENCES

1) Desgranges, C., Wolf, H., De-Thé, G., Shanmugaratnam, K., Cammoun, N., Ellouz, R., Klein, G., Lennert, K., Mundz, N. and zur Hausen, H. Nasopharyngeal carcinoma. X. Presence of Epstein-Barr genomes in separated epithelial cells of tumors in patients from Singapore, Tunisia, and Kenya. *Int. J. Cancer*, **16**, 7–15 (1975).

2) Desgranges, C., Bornkamm, G. W., Zeng, Y., Wang, P. C., Zhu, J. C., Shang, M. and De-Thé, G. Detection of Epstein-Barr viral DNA internal repeats in the nasopharyngeal mucosa of Chinese with IgA/EBV-specific antibodies. *Int. J. Cancer,* **29,** 87–91 (1982).
3) Raab-Traub, N., Flynn, K., Pearson, G., Huang, A., Levine, P., Lanier, A. and Pagano, J. The differentiated form of nasopharyngeal carcinoma contains Epstein-Barr virus DNA. *Int. J. Cancer,* **39,** 25–29 (1987).
4) Raab-Traub, N. and Flynn, K. The structure of the termini of Epstein-Barr virus as a marker of clonal cellular proliferation. *Cell,* **47,** 883–889 (1986).
5) Henle, W., Henle, G., Andersson, J., Ernberg, I., Klein, G., Horwitz, C. A., Marlund, G., Rymo, L., Wellinder, C. and Straus, E. S. Antibody responses to Epstein-Barr virus-determined nuclear antigen(EBNA)-1 and EBNA-2 in acute and chronic Epstein-Barr virus infection. *Proc. Natl. Acad. Sci. USA,* **84,** 570–574 (1987).
6) Wang, F., Gregory, C. D., Rowe, M., Rickinson, A. B., Wang, D., Birkenbach, M., Kikutani, H., Kishimoto, T. and Kieff, E. Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23. *Proc. Natl. Acad. Sci. USA,* **84,** 3452–3456 (1987).
7) Kikuta, H., Taguchi, Y., Tomizawa, K., Kojima, K., Kawamura, N., Ishizaka, A., Sakiyama, Y., Matsumoto, S., Imai, S., Kinoshita, T., Koizumi, S., Osato, T., Kobayashi, I., Hamada, I. and Hirai, K. Epstein-Barr virus genome-positive T lymphocytes in a boy with chronic active EBV infection associated with Kawasaki-like disease. *Nature,* **333,** 455–457 (1988).
8) Zimber, U., Adldinger, H. K., Lenoir, G. M., Vuillaume, M., Knebel-Doeberitz, M. V., Laux, G., Desgranges, C., Wittman, P., Freese, U-K., Schneider, U. and Bornkamm, G. W. Geographical prevalence of two types of Epstein-Barr virus. *Virology,* **154,** 56–66 (1986).
9) Polack, A., Delius, H., Zimber, U. and Bornkamm, G. W. Two deletions in the Epstein-Barr virus genome of the Burkitt lymphoma nonproducer line Raji. *Virology,* **133,** 146–157 (1984).
10) Fähraeus, R., LiFu, Hu., Ernberg, I., Finke, J., Rowe, M., Klein, G., Falk, K., Nilsson, E., Yadav, M., Busson, P., Tursz, T. and Kallin, B. Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma. *Int. J. Cancer,* **42,** 329–338 (1988).
11) Dambaugh, T., Beisel, C., Hummel, M., King, W., Fennewald, S., Cheung, A., Heller, M., Raab-Traub, N. and Kieff, E. Epstein-Barr virus DNA. VII. Molecular cloning and detailed mapping of EBV(B95-8) DNA. *Proc. Natl. Acad. Sci. USA,* **77,** 305–309 (1980).
12) Zutter, M. M., Martin, O. J., Sale, G. E., Shulman, H. M., Fisher, L., Thomas, E. D. and Durman, D. M. Epstein-Barr virus lymphoproliferation after bone marrow transplantation. *Blood,* **72,** 520–529 (1988).
13) Cleary, M. L., Nalesnik, M. A., Shearer, W. T. and Sklar, J. Clonal analysis of transplant-associated lymphoproliferations based on the structure of genomic termini of the Epstein-Barr virus. *Blood.* **72,** 349–352 (1988).
14) Brown, N. A., Liu, C., Garcia, C. R., Wang, Y-F., Griffith, A., Sparkes, R. S. and Calame, K. L. Clonal origins of lymphoproliferative disease induced by Epstein-Barr virus. *J. Virol.,* **58,** 975–978 (1986).
15) Ishihara, S., Tawa, A., Yumura-Yagi, K., Murata, M., Hara, J., Yabuchi, H., Hirai, K. and Kawa-Ha, K. Clonal T-cell lymphoproliferation containing Epstein-Barr (EB) virus DNA in a patient with chronic active EB virus infection. *Jpn. J. Cancer Res.,* **80,** 99–101 (1989).
16) Shimakage, M., Ikegami, M., Chatani, M., Yoshino, K. and Sato, T. Serological follow-up study on the antibody levels to Epstein-Barr virus-determined nuclear antigen in patients with nasopharyngeal carcinoma after radiation therapy. *Biken J.,* **30,** 45–51 (1987).