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Advances in Brief

Cloning of *bcl-6*, the Locus Involved in Chromosome Translocations Affecting Band 3q27 in B-Cell Lymphoma[1]

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Abstract

Chromosomal translocations involving band 3q27 and various chromosomal sites, including the sites of the immunoglobulin (*Ig*) loci (14q32, 2p12, 22q11), represent recurrent aberrations in non-Hodgkin's lymphoma (NHL). In order to identify the putative protooncogene involved in these translocations, we have cloned the breakpoints from two B-cell NHL cases carrying t(3;14)(q27;q32) translocations by screening genomic DNA libraries constructed from NHL biopsy samples with immunoglobulin probes. Several recombinant phages have been obtained from each case and shown to contain sequences from both 14q32 and 3q27 by fluorescence *in situ* hybridization mapping on metaphase chromosomes. In both cases, the translocation breakpoints were found within the switch region of the *Ig* heavy-chain locus on 14q32 and within the same 3-kilobase region on 3q27. When used in Southern blot hybridization, a probe from the 3q27 region detected rearrangements in an additional five NHL cases carrying 3q27 translocations with 14q32 or other genomic sites. The same probe detected a predominant 2.4-kilobase mRNA species in several lymphoid cell lines analyzed by Northern blot hybridization. These data suggest that chromosomal breakpoints in 3q27 cluster in the proximity of a transcribed gene which represents a candidate protooncogene (*bcl-6*) involved in B-cell NHL pathogenesis.

Introduction

Nonrandom chromosomal abnormalities are found in up to 90% of patients with NHL[3] and have been shown to play an important role in lymphomagenesis by activating protooncogenes (1). Some of these translocations, which are associated with specific histological subsets of NHL, have been characterized at the molecular level. In the t(8;14), t(8;22), and t(2;8) translocations associated with Burkitt's lymphoma, L_3-type acute lymphoblastic leukemia, and acquired immunodeficiency syndrome-associated NHL, a known protooncogene, c-*myc*, was found juxtaposed to the immunoglobulin (*Ig*) loci (2, 3). In the t(14;18) translocation, which is implicated in follicular-type NHL, molecular analysis of the sequences linked to the *Ig* locus led to the identification of a novel protooncogene, *bcl-2* (4–6). The t(11;14)-(q13;q32), mainly associated with "mantle zone" lymphoma, appears to involve the juxtaposition of the *Ig* heavy-chain locus with the *bcl-1* locus, the site of the candidate protooncogene PRAD-1/cyclin D1 (7, 8). These well characterized chromosome translocations are associated, however, with only a fraction of NHL cases, while a number of other recurrent translocations remain to be characterized for their genetic components.

One important example of such cytogenetic abnormalities is represented by various alterations affecting band 3q27. This region is involved in translocations with various chromosomal sites including but not limited to those carrying the *Ig* heavy- (14q32) or light- (2p12, 22q11) chain loci (9, 10). Overall, 3q27 breakpoints are detectable in 7–12% of B-cell NHL cases by cytogenetic analysis, with t(3;22)-(q27;q11) being the most frequent type detectable in 4–5% of NHL (9). The clinicopathological relevance of 3q27 breakpoints is underscored by its consistent association with diffuse-type NHL, a frequent and clinically aggressive subtype for which no specific molecular lesion has yet been identified (9).

The recurrence of 3q27 breakpoints in NHL has prompted a search for the corresponding protooncogene. We report here the cloning of clustered 3q27 breakpoints from two NHL cases carrying t(3;14)-(q27;q32) translocations and the identification of genomic rearrangements within the same breakpoint region in additional NHL cases carrying translocations involving 3q27. Within the same region, a transcriptional unit has been identified, which represents the candidate protooncogene (*bcl-6*) associated with 3q27 translocations in B-NHL.

Materials and Methods

DNA Extraction and Southern Blot Analysis. Total genomic DNA was purified from frozen tumor biopsies by cell lysis, proteinase K digestion, "salting-out" purification, and ethanol precipitation as described previously (11). Southern blot hybridization analysis was performed in 50% formamide, 3× standard saline-citrate, 10× dextran sulfate-5× Denhardt's solution-0.5% sodium dodecyl sulfate at 37°C for 16 h. Filters were washed in 0.2× standard saline-citrate-0.5% sodium dodecyl sulfate at 60°C for 2 h. DNA probes were ^{32}P-labeled by the random priming method (12).

DNA Probes. The following probes were used for Southern blot analysis of *Ig* gene rearrangements: (*a*) (J_H) probe: 6.6-kilobase *Bam*HI/*Hind*III fragment from the human *Ig* heavy-chain (Ig_H) locus (13); (*b*) (C_μ) probe: 1.3-kilobase *Eco*RI fragment containing the first two exons of human C_μ (13).

Genomic Cloning. Genomic libraries from NHL cases SM1444 and KC1445 were constructed by partial *Sau*3A restriction digestion of genomic DNA and ligation of gel-purified 15–20 kilobase fractions into Lambda-Gem-11 phage vector (Promega). Library screening was performed by plaque hybridization using the C_μ probe.

FISH Analysis. Phage DNA was labeled with biotin-14-dATP by nick translation and hybridized to metaphase spreads from normal human lymphocytes as described (14). To visualize the hybridization signal and the corresponding bands sequentially under the microscope, the slides were stained and counterstained with propidium iodide and 4′,6″-diamidino-2-phenylindole, respectively.

Northern Blot Hybridization Analysis. RNAs from several human cell lines were extracted by the guanidine isothiocyanate method (15). For Northern blot analysis, RNA samples were electrophoresed through 0.9% agarose-2.2 M formaldehyde gels and then transferred to nitrocellulose filters. Hybridization and washing were performed as described for Southern blot analysis.

Results

DNA was extracted from tumor tissue of two cases (SM1444 and KC1445) of IgM-producing, diffuse-type B-cell NHL carrying the t(3;14)(q27;q32) translocation. Since the involvement of the Ig_H locus was suspected based on the 14q32 breakpoint, SM1444 and KC1445 DNAs were first analyzed by Southern blot hybridization using combinations of enzymes and probes specific for the J_H and C_μ regions of the Ig_H locus (13). In both cases, digestion by *Bam*HI showed rearranged fragments containing J_H sequences (Fig. 1). Subsequent hy-

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[3] The abbreviations used are: NHL, non-Hodgkin's lymphoma; FISH, fluorescence *in situ* hybridization.

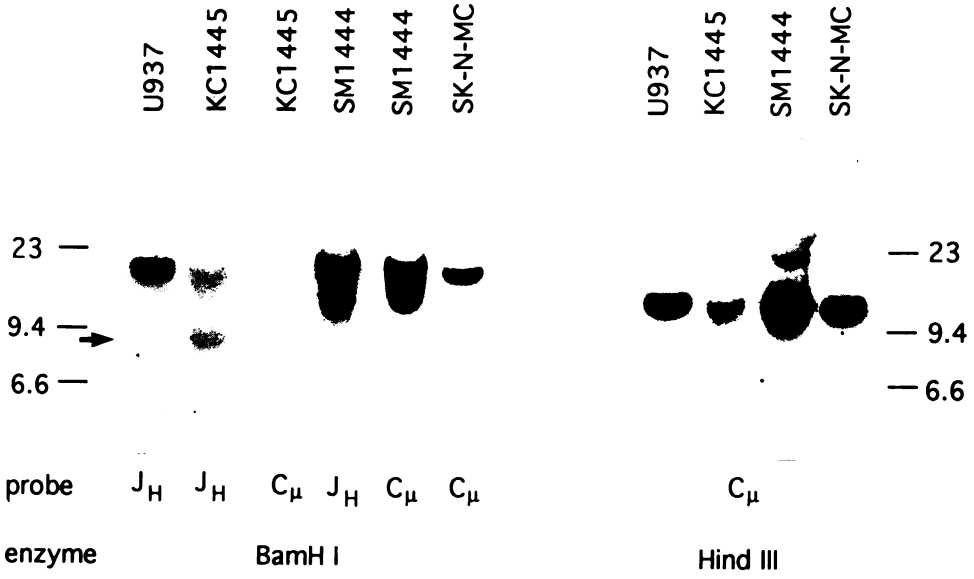

Fig. 1. Immunoglobulin gene rearrangement analysis of KC1445 and SM1444 DNA. DNA extracted from the cell lines U937 (monocytic leukemia) and SK-N-MC (neuroblastoma) were used as controls for nonrearranged, germ-line *Ig* genes. *Left*: the *arrow* on the *left* points to the rearranged J_H fragment which does not contain C_μ sequences in KC1445 DNA; the *two arrows* on the *right* point to the two distinct fragments containing J_H or C_μ sequences in SM1444 DNA.

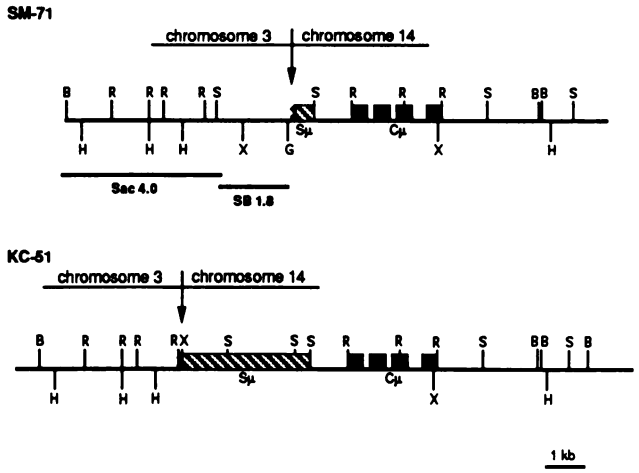

Fig. 2. Molecular cloning of the chromosomal breakpoints from two NHL cases with t(3;14). Illustrated are the maps of two representative phage clones spanning the breakpoint regions in case SM1444 (SM-71) and KC1445 (KC-51). ——, chromosome 14 portions of the phage inserts; ▧, switch sequences; ■, C_μ exons. *Vertical arrows*, junctions of chromosome 3 and 14 sequences. The probes used for Southern (Fig. 4) and Northern (Fig. 5) analysis are illustrated below the SM-71 map. Restriction enzyme sites are: *B*, *Bam*HI; *H*, *Hind*III; *R*, *Eco*RI; *G*, *Bgl*II; *S*, *Sac*I. *kb*, kilobase.

bridizations to the C_μ probe showed, in each case, that the rearranged fragment containing J_H sequences was not linked to C_μ sequences (see failure of the C_μ probe to hybridize to the same rearranged *Bam*HI fragment detected by J_H; Fig. 1) as would be expected for a physiologically rearranged Ig_H allele in IgM-producing cells. In addition, in both cases, digestion with *Hind*III and hybridization with C_μ detected a rearranged fragment, a finding inconsistent with either germ-line or physiologically rearranged Ig_H genes, since both *Hind*III sites flanking C_μ sequences are not involved in *V-D-J* rearrangements (13). The observed pattern is, however, consistent with chromosomal breakpoints located within C_μ switch sequences, as observed previously in several cases of chromosomal translocations involving the Ig_H locus (2, 16–18).

On the basis of this analysis, we cloned the C_μ containing fragments from each case by screening genomic libraries constructed from SM1444 and KC1445 DNAs using the C_μ probe. Restriction mapping and hybridization analysis of several phage clones led to the identification of recombinant phages from each library which contained C_μ sequences linked to sequences unrelated to the Ig_H locus (see Fig. 2 for maps of representative phage clones). The *Ig* portions of the phage inserts overlapped along the C_μ region extending 5′ into the switch region where alignment with the restriction map of the normal *Ig* heavy-chain locus was lost. The location of the breakpoint within C_μ switch sequences was confirmed for case SM1444 by DNA sequence analysis of the breakpoint junction of phage SM-71 (data not shown), which revealed the presence of the repeated motifs typical of the Ig_H switch regions on the chromosome 14 side (19). The *Ig*-unrelated portions of phage SM-71 and KC-51 also overlapped with each other in their restriction maps, suggesting that they were derived from the same genomic region. This notion is further supported by the fact that probe Sac 4.0 derived from SM-71 was able to hybridize to the corresponding region of KC-51 in Southern blot analysis (not shown).

To determine the chromosomal origin of the *Ig*-unrelated sequences, a recombinant phage (SM-71) derived from case SM1444, was used as a probe in FISH analysis on metaphase chromosome spreads from mitogen-stimulated normal blood lymphocytes. The phage probe hybridized specifically to chromosome 14q32 as well as to chromosome 3q27 (Fig. 3), indicating that the recombinant phage insert contained one of the two chromosomal junctions of the reciprocal t(3;14) translocation. Thus, taken together, the results of cloning and FISH analysis established that, in both NHL cases studied, the chromosomal translocation has linked sequences within the switch region of the C_μ locus to sequences from band 3q27, consistent with the cytogenetic description of the t(3;14)(q27;q32) translocation. In the two NHL cases studied, the breakpoints on 3q27 were located within 3 kilobases of the same genomic locus, which was termed *bcl-6*.

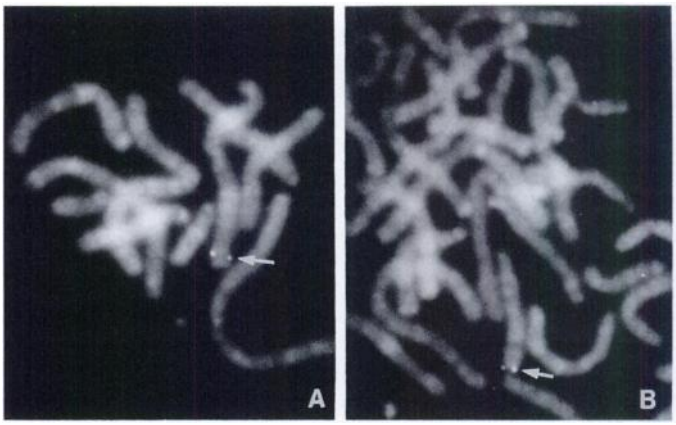

Fig. 3. Localization of phage SM-71 sequences to chromosomes 3 and 14 by fluorescence *in situ* hybridization. Consistent hybridization signals at 3q27 (*arrow* in *A*) and 14q32 (*arrow* in *B*) demonstrated that the insert is derived from the translocation junction.

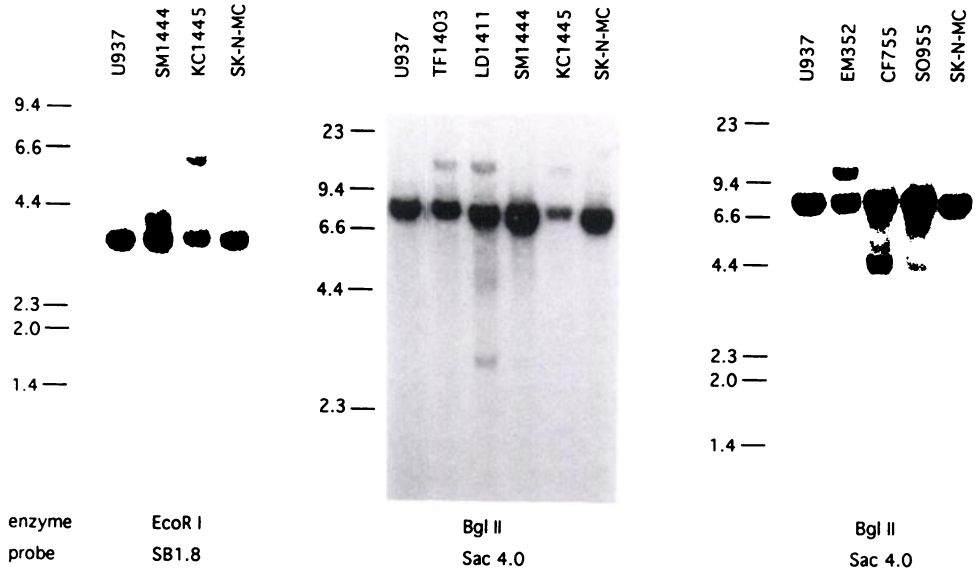

Fig. 4. Southern blot hybridization analysis of *bcl-6* rearrangements in NHL carrying 3q27 breakpoints. The probes used are illustrated in Fig. 2. U937 and SK-N-MC DNAs are used as germ-line controls since their hybridization pattern was identical to the one observed in a panel of 19 control DNAs tested. The detected cytogenetic abnormalities affecting 3q27 in each case are: KC1445, t(3;14)(q27; q32); SM1444, t(3;14)(q27;q32); TF1403, t(3;14)(q27;q32); LD1411, t(3;14)-(q27;q32); EM352, t(3;22)(q27;q11); CF755, t(3;12)(q27;q11); SO955, der(3) t(3;5)(q27;q31).

In order to determine whether 3q27 breakpoints in additional NHL cases were also located within the cloned portion of the *bcl-6* locus, we looked for *bcl-6* rearrangements in a total of 19 NHL cases carrying 3q27 breakpoints, including 4 (2 cloned cases and 2 additional ones) carrying t(3;14)(q27;q32) as well as 15 cases carrying 3q27 translocations involving regions other than 14q32. Southern blot hybridization using probes derived from phage SM-71 (see Fig. 2) detected rearranged fragments in *Eco*RI- and/or *Bgl*II-digested DNA in 7 of 19 cases studied, including all 4 t(3;14) cases as well as 3 cases with other types of translocations (see Fig. 4 for cytogenetic description of the cases and representative results). These results indicate that heterogeneous 3q27 breakpoints cluster in a fairly restricted region within *bcl-6* independently of the partner chromosome involved in the translocation.

Next we investigated whether the *bcl-6* locus adjacent to the chromosomal breakpoints contained a transcriptional unit. Probe Sac 4.0 (see Fig. 2) was used to detect RNA expression in several human cell lines by Northern blot analysis. A major 2.4-kilobase RNA species was readily detectable in two B-cell-derived cell lines tested, while a relatively less abundant 4.4-kilobase species is present in CB33 only.

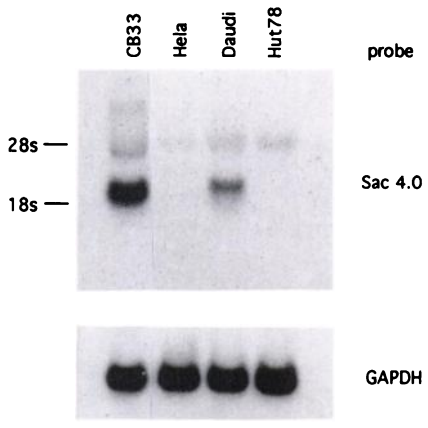

Fig. 5. Identification of the *bcl-6* transcriptional unit. Total RNA (15 μg) isolated from the indicated human cell lines was analyzed by Northern blot hybridization using the Sac 4.0 probe (see Fig. 2). CB33, Epstein-Barr virus-immortalized human B lymphoblastoid cell line; HeLa, human cervical carcinoma cell line; Daudi, human Burkitt's lymphoma cell line; Hut78, human T-cell leukemia cell line. Hybridization of the same filter to a mouse glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe is shown as control for RNA amount loaded in each lane. The faint band comigrating with 28S RNA in all the lanes may be the result of cross-hybridization with rRNA sequences. The high molecular weight band detectable in CB33 may represent an unspliced gene of *bcl-6* RNA.

No hybridization was detected in a T-cell-derived cell line (HUT 78) or in HeLa cells (Fig. 5). This result indicates that 3q27 sequences immediately adjacent to the chromosomal breakpoint cluster are part of a gene (*bcl-6*) which is expressed in cells of the B-lineage.

Discussion

This study reports the identification and cloning of a genomic region, *bcl-6*, involved in recurrent chromosomal translocations affecting band 3q27 in NHL. The region is defined by the clustered position of breakpoints in seven NHL cases carrying 3q27 translocations involving either Ig_H or several other loci. A more precise definition of the *bcl-6* locus and of the frequency of its involvement in NHL requires cloning and characterization of additional *bcl-6* sequences and the study of additional tumor cases. Nevertheless, the finding that various translocation partner chromosomes have been joined to the same region on chromosome 3 in cytogenetically heterogeneous NHL cases supports the notion that rearrangement of the *bcl-6* locus may represent the critical common denominator of translocations involving 3q27.

The second finding of this study is that the *bcl-6* locus contains a gene which is expressed in B-cells. It is not clear at this stage whether the chromosomal breakpoints directly truncate coding or regulatory sequences of *bcl-6* or whether the gene remains intact with its regulation overridden by transcriptional control motifs juxtaposed by the translocation. The clustering of breakpoints in the seven studied NHL cases suggests, however, that *bcl-6* may be a protooncogene which can contribute to NHL pathogenesis upon activation by chromosomal translocation. Results of this study will allow elucidation of the normal structure and function of the *bcl-6* gene in order to understand the pathogenetic consequences of chromosomal translocation of *bcl-6* and its role in lymphomagenesis.

References

1. Gaidano, G., and Dalla-Favera, R. Oncogenes and tumor suppressor genes. *In:* D. M. Knowles (ed.), Neoplastic Hematopathology, pp. 245–261. Baltimore: Wilkins & Wilkins, 1992.
2. Dalla-Favera, R., Bregni, M., Erickson, J., Patterson, D., Gallo, R. C., and Croce, C. M. Human c-*myc* oncogene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc. Natl. Acad. Sci. USA, *79:* 7824–7827, 1982.
3. Taub, R., Kirsch, I., Morton, C., Lenoir, G. M., Swan, D., Tronick, S., Aaronson, S., and Leder, P. Translocation of c-*myc* gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc. Natl. Acad. Sci. USA, *79:* 7837–7841, 1982.
4. Bakhshi, A., Jensen, J. P., Goldman, P., Wright, J. J., McBride, O. W., Epstein, A. L., and Korsmeyer, S. J. Cloning the chromosomal breakpoint of t(14;18) human lym-

phomas: clustering around J_H on chromosome 14 and near a transcriptional unit on 18. Cell, *41:* 889–906, 1985.
5. Tsujimoto, Y., Cossman, J., Jaffe, E., and Croce, C. M. Involvement of the *Bcl-2* gene in human follicular lymphoma. Science (Washington DC), *228:* 1440–1443, 1985.
6. Cleary, M. L., and Sklar, J. Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc. Natl. Acad. Sci. USA, *82:* 7439–7444, 1985.
7. Motokura, T., Bloom, T., Goo, K. H., Juppner, H., Ruderman, J. V., Kronenberg, H. M., and Arnold, A. A novel cyclin encoded by a *bcl-1* linked candidate oncogene. Nature (Lond.), *350:* 512–514, 1991.
8. Raffeld, M., and Jaffe, E. S. *Bcl-1*, t(11;14), and mantle zone lymphomas. Blood, *78:* 259–261, 1991.
9. Offit, K., Jhanwar, S., Ebrahim, S. A. D., Filippa, D., Clarkson, B. D., and Chaganti, R. S. K. t(3;22)(q27;q11): A novel translocation associated with diffuse non-Hodgkin's lymphoma. Blood, *74:* 1876–1879, 1989.
10. Bastard, C., Tilly, H., Lenormand, B., Bigorgne, C., Boulet, D., Kunlin, A., Monconduit, M., and Piguet, H. Translocations involving band 3q37 and Ig gene regions in non-Hodgkin's lymphoma. Blood, *79:* 2527–2531, 1992.
11. Miller, S. A., Dykes, D. D., and Polesky, H. F. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res., *16:* 1215–1218, 1988.
12. Feinberg, A. P., and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem., *132:* 6–13, 1983.
13. Ravetch, J. V., Siebenlist, U., Korsmeyer, S., Waldman, T., and Leder, P. Structure of the human immunoglobulin μ locus: characterization of embryonic and rearranged J and D regions. Cell, *27:* 583–591, 1981.
14. Rao, P. H., Murty, V. V. V. S., Gaidano, G., Hauptschein, R., Dalla-Favera, R., and Chaganti, R. S. K. Subregional localization of 20 single-copy loci to chromosome 6 by fluorescence *in situ* hybridization. Genomics, in press, 1993.
15. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry, *18:* 5294–5299, 1979.
16. Peschle, C., Mavilio, F., Sposi, N. M., Giampaolo, A., Care, A., Bottero, L., Bruno, M., Mastroberardino, G., Gastaldi, R., Testa, R., Alimena, M. G., Amadori, S., and Mandelli, F. Translocation and rearrangement of c-*myc* into immunoglobulin α heavy chain locus in primary cells from acute lymphocytic leukemia. Proc. Natl. Acad. Sci. USA, *81:* 5514–5518, 1984.
17. Showe, L. C., Ballantine, M., Nishikura, K., Erikson, J., Kaji, H., and Croce, C. M. Cloning and sequencing of a c-*myc* oncogene in a Burkitt's lymphoma cell line that is translocated to a germ line α switch region. Mol. Cell. Biol., *5:* 501–509, 1985.
18. Neri, A., Barriga, F., Knowles, D. M., Magrath, I., and Dalla-Favera, R. Different regions of the immunoglobulin heavy chain locus are involved in chromosomal translocations in distinct pathogenetic forms of Burkitt lymphoma. Proc. Natl. Acad. Sci. USA, *85:* 2748–2752, 1988.
19. Rabbitts, T. H., Forster, A., and Milstein, C. P. Human immunoglobulin heavy chain genes: evolutionary comparisons of Cμ, Cδ and Cγ genes and associated switch sequences. Nucleic Acids Res., *9:* 4509–4524, 1981.