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Molecular localization of the t(11;22)(q24;q12) translocation of Ewing sarcoma by chromosomal *in situ* suppression hybridization

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ABSTRACT **Chromosome translocations are associated with a variety of human leukemias, lymphomas, and solid tumors. To localize molecular markers flanking the t(11;22)(q24;q12) breakpoint that occurs in virtually all cases of Ewing sarcoma and peripheral neuroepithelioma, high-resolution chromosomal *in situ* suppression hybridization was carried out using a panel of cosmid clones localized and ordered on chromosome 11q. The location of the Ewing sarcoma translocation breakpoint was determined relative to the nearest two cosmid markers on 11q, clones 23.2 and 5.8, through the analysis of metaphase chromosome hybridization. By *in situ* hybridization to interphase nuclei, the approximate physical separation of these two markers was determined. In both Ewing sarcoma and peripheral neuroepithelioma, cosmid clone 5.8 is translocated from chromosome 11q24 to the derivative chromosome 22 and a portion of chromosome 22q12 carrying the leukemia inhibitory factor gene is translocated to the derivative chromosome 11. The physical distance between the flanking cosmid markers on chromosome 11 was determined to be in the range of 1000 kilobases, and genomic analysis using pulsed-field gel electrophoresis showed no abnormalities over a region of 650 kilobases in the vicinity of the leukemia inhibitory factor gene on chromosome 22. This approach localizes the Ewing sarcoma breakpoint to a small region on chromosome 11q24 and provides a rapid and precise technique for the molecular characterization of chromosomal aberrations.**

Consistent and specific chromosome translocations have been associated with a number of human malignancies including leukemias, lymphomas, and solid tumors and may be intimately involved in the molecular pathogenesis of the associated disorders (1–5). Molecular studies of translocations in solid tumors lag far behind the study of leukemias due to the technical difficulties of chromosome analysis in tissue samples (1, 2). Human chromosome 11 contains several sites of chromosome rearrangement associated with tumors including t(11;22)(q13;q13) rearrangements involving the *BCL1* (breakpoint cluster 1) locus in B-cell chronic lymphocytic leukemia, B-cell non-Hodgkin lymphoma, and multiple myeloma (6), t(4;11)(q21;23) associated with infantile acute lymphoblastic leukemia (7), and t(9;11)(p22;q23) and t(11;19)(q23;p13) in cases of acute monocytic leukemia (8). The t(11;22)(q24;q12) translocations of Ewing sarcoma (ES), peripheral neuroepithelioma (PNE), and Askin tumor appear to be cytogenetically identical and represent the best described and most consistent chromosome abnormalities associated with solid tumors (9, 10). Both ES and PNE are small round-cell tumors occurring in the trunk or extremities and may arise through transformation of neuroectodermally derived cells (11). ES cells in culture express neuroectoderm-associated antigens (12, 13), and ES tumors share a number of histological and immunocytochemical similarities with other tumors derived from neural crest (11). ES and PNE have indistinguishable patterns of expression of various protooncogenes (14) and may represent extremes of a spectrum of tumor cell types ranging from more differentiated (PNE) to least differentiated (ES) neuroectodermal cell types (11).

Previous molecular analysis of the ES and PNE translocations has been inhibited by the lack of a sufficient density of molecular probes to allow precise localization and molecular cloning of the site of translocation. Pulsed-field gel analysis using a limited number of randomly selected and localized molecular probes has thus far failed to reveal the site of translocation (15). Here, we have applied *in situ* hybridization of a panel of nonisotopically labeled cosmid clones previously mapped to chromosome 11 (16) and suppression hybridization to eliminate the signal from repetitive sequences (16) to localize the ES and PNE breakpoint between two closely spaced DNA markers, cosmids 23.2 and 5.8. Use of these clones for high-resolution analysis of ES and PNE interphase nuclei allowed the localization of the translocation breakpoint on chromosome 11 between the nearest two flanking cosmids, to a region that approximates 1 megabase (Mb). In addition, the gene encoding the leukemia inhibitory factor (LIF), shown to map to human chromosome 22 in the vicinity of the ES breakpoint (17), was found to be translocated to the ES and PNE derivative chromosome 11 to the immediate vicinity of the most centromeric flanking cosmid marker. Since LIF has been shown to suppress *in vitro* proliferation of myeloid leukemia cell lines (18) and to prevent differentiation of embryonic cells in culture (19), a chromosome translocation in the vicinity of this gene might be sufficient to induce oncogenesis. However, pulsed-field gel analysis demonstrated no abnormalities in a 650-kb region surrounding this locus. Chromosomal *in situ* suppression hybridization (CISSH) coupled with panels of landmark cosmid clones will allow rapid mapping and molecular cloning of the ES and PNE breakpoints as well as differential diagnosis within the group of mixed round-cell tumors, where only ES and PNE show this cytogenetic abnormality (20).

MATERIALS AND METHODS

Cell Lines and Cosmid Clones. Human tumor cell lines TC71 and 6674, derived from ES, and TC32, derived from peripheral neuroepithelioma (21), were obtained from T. Triche (Los Angeles Children's Hospital) and were shown to retain the previously described t(11;22)(q24;q12) translocation. A human fibroblast line, CRL 1634, with a normal karyotype was obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Cosmid clones mapping to 11q13–11qter (16, 22, 23), as well as those containing the *THY1* (24, 25), *CD3* (26), and *ETS1* (27) genes, have been described. Cosmid LIF3E2II, carrying the human LIF gene (18, 19, 28), was isolated from a human genomic cosmid library (25). Cosmid Hu-lambda 9, containing the immuno-

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Abbreviations: CISSH, chromosomal *in situ* suppression hybridization; ES, Ewing sarcoma; FLpter, fractional chromosomal length from end of short arm; LIF, leukemia inhibitory factor; PFGE, pulsed-field gel electrophoresis; PNE, peripheral neuroepithelioma.

globulin λ constant-region gene, has been described (29). Probes for *in situ* hybridization were labeled by primer extension using random oligomers (30) in the presence of the biotinylated nucleotides bio-11-dUTP and bio-11-dCTP (Enzo Biochemicals) as described (16).

Slide Preparation and CISSH. Metaphase chromosomes were prepared from actively growing cells by mitotic blockage and spread on microscope slides (31). Interphase nuclei were prepared from cells harvested 5–6 days after complete confluency to obtain a relatively pure population of cells in the G_1 phase of the cell cycle (32). Hybridization and suppression reactions were carried out by modifications of published procedures (16, 33). Hybridization was visualized by treating the slides with fluoresceinated avidin and biotinylated goat anti-avidin (Vector Laboratories), both at 5 μg/ml (16, 33). Images were produced using a laser scanning confocal microscope (Bio-Rad MRC 500), and narrow-band-pass filters were used to obtain separate images for fluorescein isothiocyanate (550 nm) and propidium iodide (610 nm), which were then superimposed electronically.

Pulsed-Field Gel Electrophoresis (PFGE). DNA was obtained from the fibroblast cell line CRL 1634, from normal peripheral blood lymphocytes, and from tumor cell lines TC32, TC71, and 6674 in agarose plugs (34), digested with *Not* I, *Bss*HII, *Sfi* I, and *Mlu* I, and analyzed using the hexagonal contour-clamped homogeneous electric field (HEX-CHEF) system (ref. 35; CBS Scientific, Del Mar, CA).

RESULTS

CISSH using nonisotopically labeled cosmid clones (16) allows rapid isolation of DNA markers flanking chromosome translocation breakpoints so that molecular cloning may proceed by cosmid "walking" (25). To precisely locate the 11;22 translocation associated with ES and PNE, we used a set of ordered cosmid DNA markers previously mapped on chromosome 11q by CISSH (16). These ordered cosmid

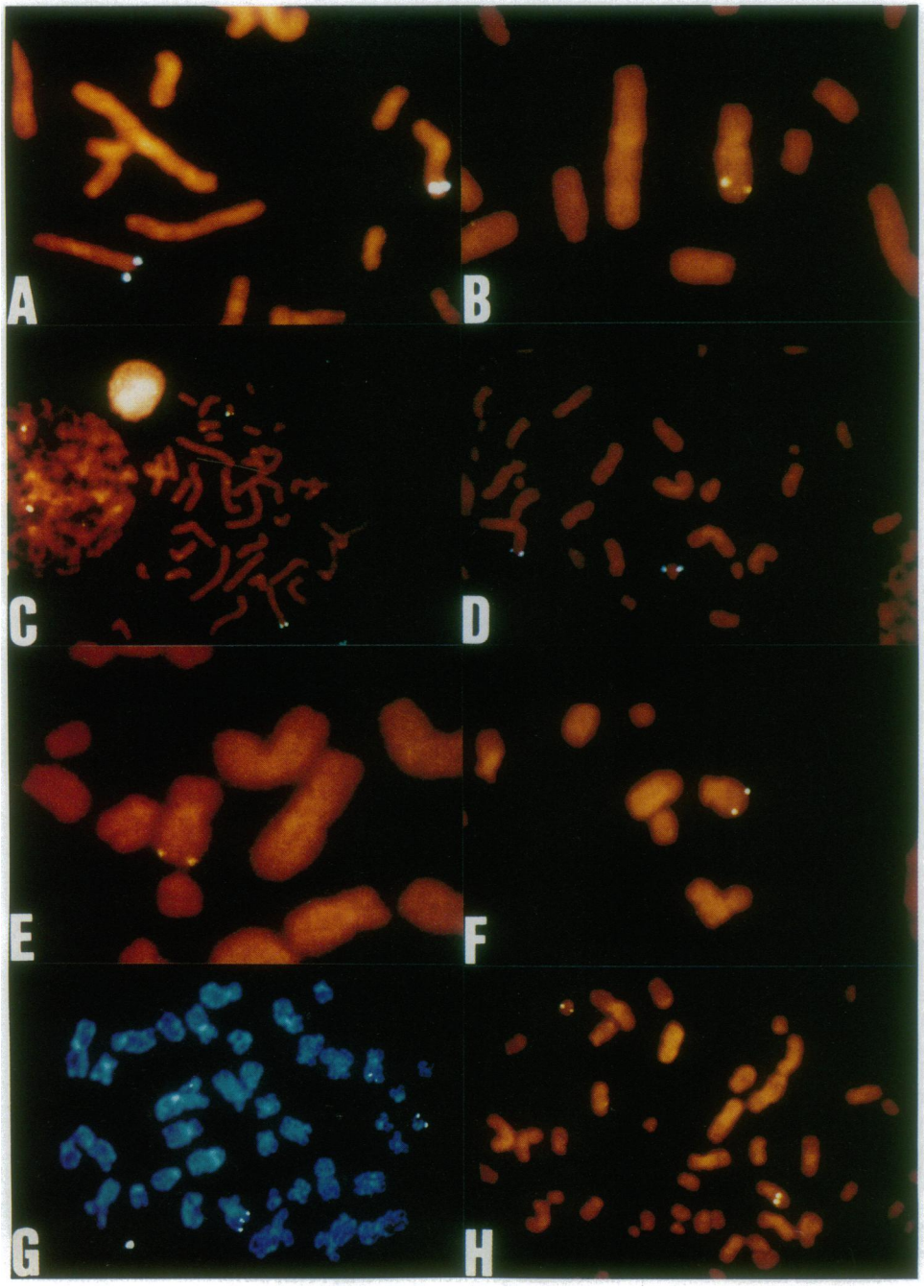

FIG. 1. CISSH with cosmid DNA to metaphase chromosomes from ES and PNE cell lines. Cosmid DNA was prepared and biotinylated as described (16) and detected using fluoresceinated avidin, and chromosomes were visualized by counterstaining with propidium iodide. Data were collected and images were prepared using a Bio-Rad MRC500 confocal microscope; images were electronically enlarged for analysis. (*A*) Hybridization of cosmid clone 23.2, previously mapped to 11q24–qter (16), to metaphase chromosomes from the ES cell line TC71. The normal chromosome 11 is on the left and the elongated derivative 11;22 on the right. (*B*) Cosmid clone 23.2 hybridized to a derivative 11;22 from ES cell line TC71. (*C*) Hybridization of cosmid clone 5.8, previously localized to 11qter (16), to metaphase chromosomes from ES cell line TC71. The normal chromosome 11 is seen in the lower portion of the field, and the derivative chromosome 22 in the upper part of the field. (*D*) CISSH of clone 5.8 to PNE cell line TC32. Hybridization to the normal chromosome 11 is seen at left and to the derivative chromosome 22 at center of the field. (*E*) Enlargement of normal chromosome 11 from ES cell TC71 hybridized with clone 5.8. (*F*) Hybridization of clone 5.8 to derivative chromosome 22 from ES cell line TC71. (*G*) Simultaneous hybridization of cosmid clones 5.8 and 23.2 to metaphase chromosomes from ES cell lines TC71. The normal chromosome 11 (lower) shows four spots of hybridization, two on each sister chromatid; the derivative chromosome 22 (middle right) shows two hybridization spots and the derivative chromosome 11 (upper right) shows two hybridization spots. (*H*) CISSH of cosmid clone LIF3E2II, containing the human LIF gene, to metaphase chromosomes from ES cell line TC71. Hybridization to the normal chromosome 22 (upper left) and derivative chromosome 11 (center) is evident.

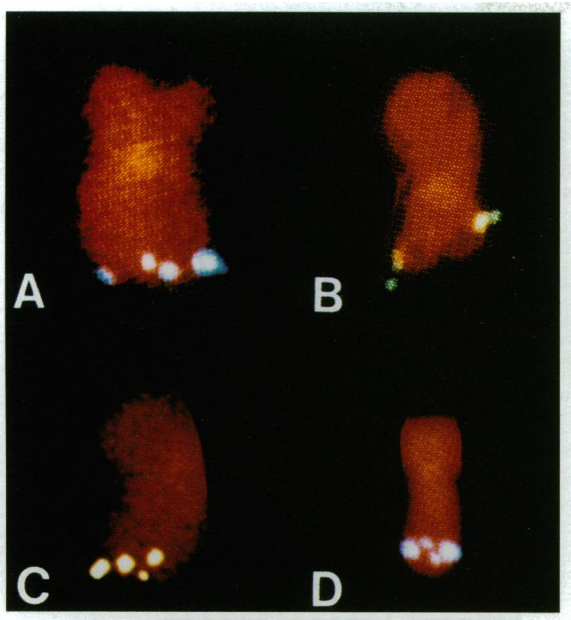

FIG. 2. CISSH of cosmid clones flanking the ES and PNE breakpoints to normal and derivative chromosomes 11. Images were collected and analyzed using confocal microscopy. (*A–C*) Normal chromosome 11 from three different metaphases of the cell line TC71 hybridized simultaneously with cosmid clones 23.2 and 5.8, flanking the translocation breakpoint. (*D*) Derivative chromosome 11;22 from ES cell line TC71 hybridized with cosmid clones 23.2 and LI3E2II, flanking the translocation breakpoint.

clones were labeled and sequentially hybridized to metaphase chromosomes from normal, ES, or PNE cell lines, and the location of the hybridization signal on the normal and derivative chromosome 11 or 22 was determined. In the absence of traditional cytogenetic "banding," chromosomes were identified by hybridization with an additional cosmid clone, Hu-lambda 9, containing the human immunoglobulin λ constant-region gene on chromosome 22 (29), or a cosmid previously mapped to chromosome 11 (16). Hybridization to both sister chromatids of the normal or derivative chromosomes was seen in 85–90% of metaphases examined, and through electronic enlargement using a confocal laser scanning microscope, the fractional chromosomal length from the end of the short arm (FLpter; ref. 16) was determined on normal and derivative chromosomes. Consistent with previous reports, cosmids containing *ETS1*, *THY1*, and *CD3* were located centromeric to the translocation breakpoint (21). Cosmid clone 23.2, previously mapped at FLpter 0.98 (16), was present on the derivative chromosome at FLpter 0.88, a consequence of significant elongation of the chromosome due to the translocated fragment of chromosome 22 (Fig. 1). Cosmid clone 5.8, previously mapped with an FLpter of 0.98, was found to be translocated to the derivative chromosome 22 in both ES and PNE metaphases (Fig. 1).

Since clones 23.2 and 5.8 were previously shown to be separated by 1–4% of the chromosome length (≈1–6 Mb) by measurements of distance from the 11p telomere (16), more precise determination of physical separation of clones was carried out by pairwise hybridization of cosmids (Figs. 1 and 2). Clones 23.2 and 5.8 were labeled and simultaneously hybridized to normal, ES, and PNE metaphase chromosomes, demonstrating four distinct fluorescent spots on the normal chromosome 11 in 70% of metaphases examined. Two fluorescent signals were observed on each of the derivative chromosomes 11 and 22, demonstrating that the translocation separates these two closely spaced markers. The current analysis suggests that the separation between the flanking markers 23.2 and 5.8 corresponds to a physical distance in the range of 1 Mb, which is near the limit of this technique (16).

Trask *et al.* (32) and Lawrence *et al.* (36), using the Chinese hamster dihydrofolate reductase and the human dystrophin loci as models, demonstrated that fluorescence *in situ* hybridization to interphase nuclei using individual cosmid clones can be used to estimate linear distance between DNA markers spatially separated by 30–1000 kb. A linear correlation between spatial separation and linear separation on the chromosome was found in interphase nuclei in both systems, between 30 kb and 1 Mb (32, 36). Furthermore, different cell types were examined (36), yielding very similar interphase distances in the 1-Mb size range. This indicates a common level of condensation, at a given distance, for chromatin from different sources, thus supporting the derivation of approximate genomic distance from interphase distance on different chromosomes. To estimate the molecular distance separating the two cosmid probes flanking the ES and PNE breakpoints, we carried out *in situ* hybridization using interphase nuclei from normal human fibroblasts and ES and PNE cell lines (Fig. 3). Clear individual positions of hybridization were observed in virtually all nuclei examined, and electronic enlargement was used to determine the average separation of hybridization signals on 20–30 individual nuclei. Since the average separation of hybridization signals for clones 23.2 and 5.8 ranged from 1 to 1.4 μm, these clones are expected to be separated by a genomic segment that approximates 0.8–1.5 Mb on chromosome 11, using as a standard the analysis of Trask *et al.* (32) and Lawrence *et al.* (36). Analysis of nuclei in ES and PNE cells demonstrated a separation of the two probes corresponding to the chromosome translocation (Fig. 3) in 40–50% of nuclei. Quantitative variations in hybridization signals in the remaining nuclei corresponded to aneuploidy and to the presence of cells in G_2/M, characteristic of cells derived from solid tumors.

It is tempting to consider that genes whose normal pattern of expression influences cell growth might be involved in

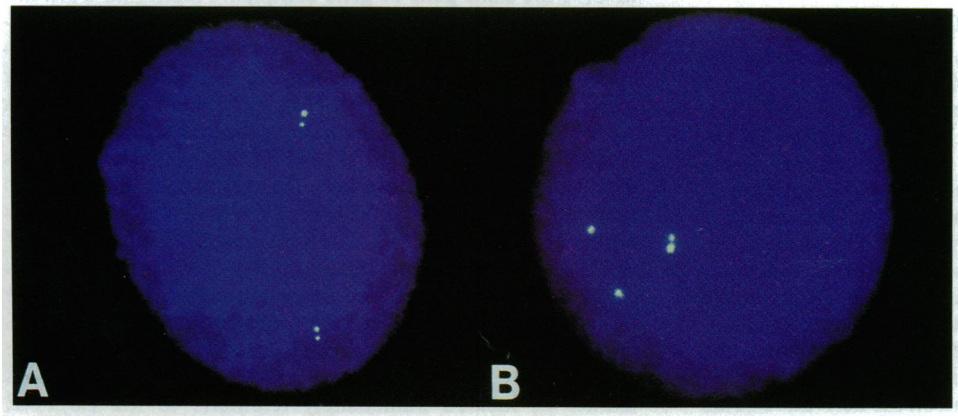

FIG. 3. CISSH of clones 23.2 and 5.8 to interphase nucleus from normal human fibroblast line CRL 1634 (*A*) and from ES cell line TC71 (*B*). DNA probe preparation and hybridization to interphase nuclei were carried out as described (16, 32). Images were collected and electronically enlarged using confocal microscopy. Cosmids were labeled with fluorescein and nuclei were counterstained with propidium iodide. Colors in these photographs were electronically altered.

chromosome translocations associated with tumors. The gene encoding LIF, an interleukin involved in the regulation of cell growth, was mapped to chromosome 22q12 (17), in the cytogenetic vicinity of the ES translocation breakpoint. To determine whether the translocation occurred near this gene, we isolated a series of cosmid clones containing the LIF gene from a human genomic cosmid library by using synthetic probes based on published sequences (28). To determine the precise chromosomal location relative to other cosmid markers, CISSH was carried out using metaphase chromosomes from normal human, ES, and PNE cells. The LIF gene mapped to the normal chromosome 22 with an FLpter of 0.60, corresponding to the band 22q12 (Fig. 1). When the LIF cosmid was used in hybridization to metaphases from ES cell lines TC71 and 6647, two hybridization signals were found on the normal chromosome 22 and hybridization signals were observed on the derivative chromosome 11 at FLpter 0.88. Thus, consistent with recent analysis of somatic cell hybrids (37), the LIF gene is located distal to the t(11;22) breakpoint on chromosome 22 and is relocated onto the derivative chromosome 11 as a consequence of this chromosomal rearrangement. Identical hybridization positions were observed on metaphases from the PNE cell line TC32 (data not shown), suggesting that the ES and PNE translocations are in the same relative location. To determine the location and distance of the translocated LIF gene to the cosmids flanking the chromosome 11 breakpoint, simultaneous CISSH analysis using the LIF cosmid and clone 23.2 was carried out with ES and PNE cell lines (Fig. 2), demonstrating four fluorescent spots located at FLpter 0.88, two on each chromatid, on the derivative chromosome 11. Metaphase and interphase analysis of distance, using the same size standard as described above, suggested that the LIF–23.2 distance on the derivative chromosome was in the range of 1 Mb.

Since the LIF gene encodes an interleukin with significant developmental effects on cell growth, we investigated whether the LIF gene would be interrupted by the translocation, carrying out PFGE to probe the region of chromosome 22 in the vicinity of LIF. DNA samples from the normal fibroblast cell line, from normal peripheral blood lymphocytes, and from ES and PNE cell lines were digested with rare-cutting restriction enzymes (*Mlu* I, *Bss*HII, *Sfi* I, *Not* I) and hybridized to a repeat-free probe prepared from cosmid LIF3E2II. Unique fragments were identified in normal, ES, and PNE DNA samples and no evidence of rearrangements occurring within these fragments was observed (Fig. 4). As cosmid LIF3E2II, from which the repeat-free probe was generated, did not contain internal *Not* I sites, these data suggest that the t(11;22) breakpoint in both ES and PNE cell lines lies outside a 650-kb *Not* I fragment spanning the LIF gene. Since the separation of the LIF gene and 23.2 on the derivative chromosome is in the range of 1 Mb, this limits the area of the breakpoint to a small genomic region.

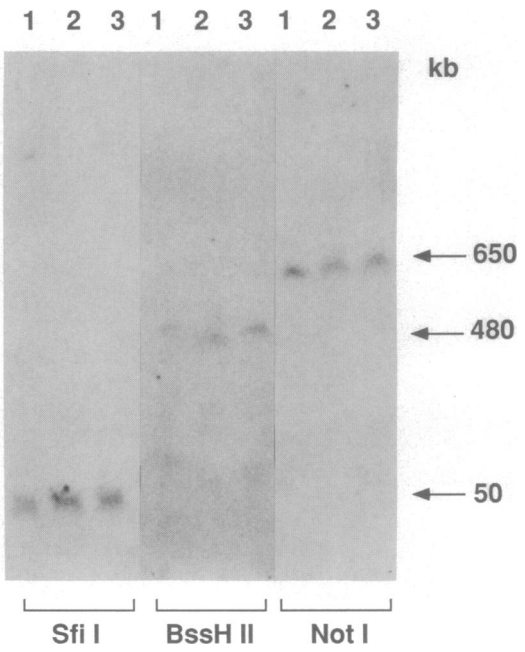

FIG. 4. Analysis of the LIF gene region on chromosome 22 by PFGE in the hexagonal contour-clamped homogeneous electric field (HEX-CHEF) configuration. DNA from human peripheral blood lymphocytes (lanes 1), ES cell line TC71 (lanes 2), and PNE cell line TC32 (lanes 3) was prepared in agarose blocks, digested with *Sfi* I, *Bss*HII, or *Not* I, separated by PFGE, transferred to nylon-backed filters, and hybridized with unique-sequence probes from cosmid clone LIF3E2II. Sizes of bands (kb) are derived from normal *Saccharomyces cerevisiae* chromosomes used as markers.

DISCUSSION

Traditional cytogenetic analysis has proven valuable for the analysis of chromosome abnormalities but allows resolution only to the limit of cytogenetic banding, about 5–30 Mb. Fluorescence *in situ* hybridization using collections of ordered cosmid clone "landmarks" provides an extremely powerful tool for extending the precision and resolution of cytogenetic analysis and for providing flanking DNA markers for direct molecular analysis of chromosome aberrations (16). This technique should prove valuable for the molecular characterization of breakpoints occurring in solid tumors. We applied this technique, coupled with a battery of chromosome-specific ordered cosmid clones (16, 22), to locate the ES and PNE translocation breakpoints to an interval of ≈1 Mb on the normal chromosome 11 and on the derivative 11;22 chromosome, and to estimate the spatial separation between the flanking markers by using measurements from interphase *in situ* hybridizations. The localization within a region of ≈1 Mb will allow long-range mapping by PFGE (35) as well as the isolation of additional DNA in cosmids (22) or yeast artificial chromosomes (YACs) (38). Since the size of cloned fragments in YAC clones is 100–700 kb (38), the localization and cloning of the breakpoint with YAC or cosmid clones will be greatly expedited.

This work results in high-resolution localization of the ES and PNE breakpoints within the 11q24 cytogenetic band and also within a standard reference map (16) of cosmid DNA markers on human chromosome 11 (Fig. 5). The breakpoint is in the vicinity of the LIF gene on chromosome 22q12, but PFGE analysis demonstrated that the LIF gene is not interrupted. The ES and PNE breakpoints are indistinguishable from each other and map to the same molecular interval even at this high level of resolution. Thus, the cytogenetic abnormalities in these two histologically distinct tumors are likely to involve the same gene or gene complex. Additional work has demonstrated that the ES/PNE translocation is distinguishable from the t(4;11), t(9;11), and t(11;19) translocations occurring in leukemias, which map to a different molecular interval on the standard map (16). Consistent with previous work, the ES/PNE translocation is located telomeric to the *NCAM*, *THY1*, *CD3*, *APOA1*, and *ETS1* c-ets-1 loci as well as newly defined cosmid markers (Fig. 5), while the t(4;11) is between *NCAM/CD3* and *THY1/ETS1* loci and the t(9;11) is also centromeric to *ETS1*. The previous localization of *ETS1* in 11q24–25 (16) and the location of the 23.2 and 5.8 markers distal to that marker, based on rough estimates of distance from metaphase mapping (16), suggest that the ES/PNE translocation breakpoint may be located in the vicinity of the 11q telomere. This work also establishes the cosmid 5.8 marker as located immediately telomeric to the ES/PNE

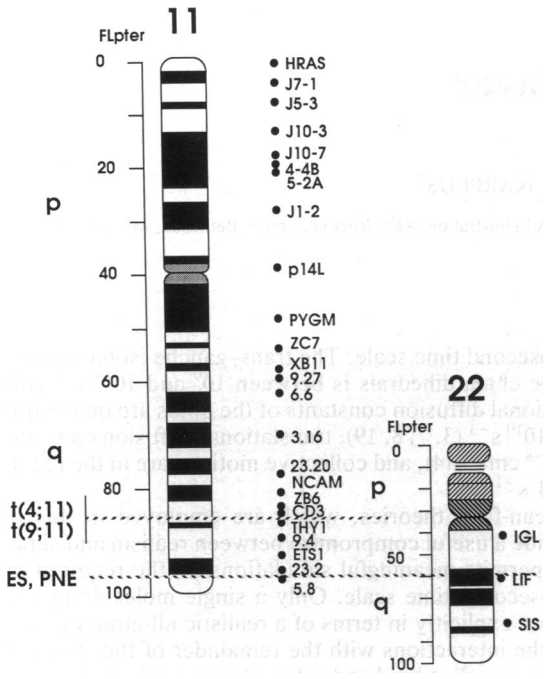

FIG. 5. ES and PNE breakpoints relative to other molecular markers on chromosomes 11 and 22 (16).

breakpoint and translocated to the derivative chromosome 22. Previous analysis located only a single DNA marker, ph2-25 (15), distal to the translocation. Based on a restriction fragment length polymorphism in this probe, the nearest DNA markers flanking the ES translocation were separated by 21% recombination on a sex-averaged map, a distance expected to correspond to about 20 Mb (39). Thus, this study significantly improves the localization of the ES/PNE breakpoint to a much smaller interval. In addition, probe selection based on random strategies has generated few DNA markers located in the interval separating the ES translocation and the 11q telomere (ref. 39 and unpublished data), and the relative scarcity of polymorphic markers in this region makes further molecular analysis on the basis of genetic linkage extremely difficult.

The use of CISSH combined with a panel of ordered cosmid probes provides a powerful tool for analysis of the t(11;22) and for the differential diagnosis of ES or PNE from other small-cell tumors such as lymphoma, neuroblastoma, rhabdomyosarcoma, and osteosarcoma. This work defines flanking DNA markers for the t(11;22) translocation of ES and PNE that may be applied to *in situ* hybridization using interphase nuclei in place of traditional banding analysis of metaphase chromosomes (Fig. 3). Thus, this work provides molecular reagents for diagnosis of cytogenetic abnormalities.

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