

## Heterogeneous Chromosomal Aberrations Generate 3' Truncations of the NFKB2/*lyt-10* Gene in Lymphoid Malignancies

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The NFKB2 (*lyt-10*) gene codes for a protein that is a member of the NF- $\kappa$ B/*rel* family of transcription factors containing a DNA-binding *rel* domain and a carboxy-terminal *ankyrin*-like domain. The NFKB2 gene represents a candidate proto-oncogene, since it has been found to be involved in a chromosomal translocation t(10;14)(q24;q32) in one case of B-cell lymphoma and in gene rearrangements in various types of lymphoid malignancies. To elucidate the structural and functional consequences of NFKB2 rearrangements, we report the molecular characterization of three novel rearranged NFKB2 genes in lymphoid tumors. In one case of multiple myeloma (MM), cloning and sequencing analysis of reciprocal breakpoint sites showed that they occurred within intron 15 of the NFKB2 gene and led to the complete deletion of the 3' portion of the gene coding for the *ankyrin* domain. Fluorescent in situ hybridization (FISH) analysis showed that the novel regions involved in the NFKB2 rearrangement originated from chromosome 7q34, thus implying the occurrence of a t(7;10)(q34;q24) reciprocal chromosomal translocation. In one case of T-cell cutaneous lymphoma (CTCL)

and in one of B-cell chronic lymphocytic leukemia (B-CLL), NFKB2 rearrangements occurred, respectively, within exons 18 and 20 of the gene and involved recombinations with distinct regions of chromosome 10q24. Molecular analysis suggested that these rearrangements may occur as a consequence of small internal chromosomal deletions. In both of these cases, the rearrangements led to specific carboxy-terminal truncations of NFKB2 generating abnormal transcripts that coded for proteins lacking portions of the *ankyrin* domain. These proteins localize in the nucleus, suggesting their constitutive activation in vivo. Overall, our results indicate that NFKB2 rearrangements in lymphoid neoplasia may occur by heterogeneous mechanisms, including internal chromosomal deletion or chromosomal translocation. The common consequence of these rearrangements appears to be the deletion of 3' sequences of NFKB2 leading to the production of carboxy-truncated constitutively nuclear proteins that may be involved in tumorigenesis.

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THE NF- $\kappa$ B/*rel* family of transcription factors is involved in the regulation of the expression of a variety of cellular and viral genes, including those that control immune responses, acute-phase reactions, and the replication of viruses, such as human immunodeficiency virus (HIV). In unstimulated cells, the NF- $\kappa$ B complexes (generally referred to as p50-p65 heterodimers) are found inactive in the cytoplasm associated with inhibitory proteins called I $\kappa$ Bs. Following a variety of stimuli, including phorbol esters, cytokines, and viruses, NF- $\kappa$ B complexes dissociate from I $\kappa$ B and translocate into the nucleus, where they bind to specific *cis*-acting consensus sequences ( $\kappa$ B sites) located in the regulatory regions of inducible genes.<sup>1,2</sup>

It is now well recognized that NF- $\kappa$ B transcription activity is mediated by heterogeneous dimeric complexes generated through the combination of a series of NF- $\kappa$ B proteins. These proteins all share a highly conserved amino-terminal domain,

the *rel* homology domain, which contains DNA-binding, dimerization, and nuclear localization functions. A first group is represented by the NF- $\kappa$ B1p105/p50<sup>3-5</sup> and its homologous NF- $\kappa$ B2p100/p52,<sup>6-8</sup> encoded by the NFKB1 and NFKB2/*lyt-10* genes, respectively. These molecules are precursor proteins (p105 and p100) that contain the amino-terminal *rel* homology domain, a poly-glycine (poly-G) hinge and a carboxy-terminal *ankyrin*-like domain.<sup>9</sup> The removal of the *ankyrin* domain, probably due to a proteolytic process mediated by the poly-G hinge, gives rise to the active DNA-binding subunits of 50-kD (NF- $\kappa$ B1p50)<sup>10,11</sup> or 52-kD (NF- $\kappa$ B2p52).<sup>12,13</sup> A second group is represented by p65/RelA,<sup>14,15</sup> c-*rel*/Rel,<sup>16</sup> and *relB*/RelB.<sup>17,18</sup> These molecules contain the amino-terminal *rel* homology domain and distinct transactivation domains within their carboxy-terminal regions, and are not subject to proteolytic processing. NF- $\kappa$ B activity is controlled by the I $\kappa$ B proteins, including I $\kappa$ B $\alpha$ /Mad-3,<sup>19</sup> I $\kappa$ B $\gamma$ ,<sup>20</sup> and the putative proto-oncogene *bcl-3*,<sup>21,22</sup> which are characterized by the presence of a domain of 5 to 7 *ankyrin*-like repeats within their carboxy-terminal regions. These proteins are involved in the cytoplasmic retention of the NF- $\kappa$ B factors and the inhibition of DNA binding activity, probably mediated by their *ankyrin* domains.<sup>23</sup> However, studies on *bcl-3* suggest that this protein does not retain NF- $\kappa$ B proteins in the cytoplasm and, therefore, may represent a distinctive member of the I $\kappa$ B family.<sup>22,24</sup>

The NFKB2/*lyt-10* gene has been identified as a novel putative proto-oncogene involved in a chromosomal translocation t(10;14)(q24;q32) in a B-cell non-Hodgkin's lymphoma (B-NHL).<sup>6</sup> In this case, the NFKB2 gene was found juxtaposed to the *Ca1* region of the immunoglobulin heavy-chain locus generating a NFKB2-*Ca1* fusion gene coding for a protein that retained the *rel* domain and lacked the entire *ankyrin* domain. This abnormal protein has been shown to bind in vitro to specific  $\kappa$ B sites, thus suggesting that the alteration of the *ankyrin* domain by genomic re-

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arrangements may represent a general mechanism of constitutive activation of the NFKB2 gene in vivo. By Southern blot analysis of a large and representative panel of lymphoid malignancies,<sup>25</sup> we have recently shown that rearrangements affecting the NFKB2 locus at 10q24 occur in approximately 2% of cases and in approximately 10% of cutaneous lymphomas.

To gain further insight into the molecular mechanisms involved in the structural alterations of the NFKB2 gene in vivo, we have cloned and sequenced the rearranged NFKB2 genes from three of our five previously reported cases. Our results confirm the direct involvement of the *ankyrin* domain in the rearrangement events and indicate that structural alterations of the NFKB2 gene may occur as a consequence of heterogeneous chromosomal aberrations in lymphoid malignancies.

### MATERIALS AND METHODS

**Molecular cloning.** Genomic libraries from cases LB363 and EB308 and case LB40 were constructed by complete digestion of genomic DNA with respectively *Bam*HI and *Eco*RI restriction enzymes, and the ligation of gel-purified fractions into the  $\lambda$ EMBL3 or  $\lambda$ EMBL4 phage vectors (Stratagene, La Jolla, CA). The individual rearranged NFKB2 clones were isolated by screening with the genomic probe Pv/H 1.4 kb representative of the amino-terminus of the NFKB2 (*rel* domain), including exons 5 to 9. For the isolation of the reciprocal product of the NFKB2 gene rearrangement in case LB363, the screening was performed using the genomic probe *Pst*I 1.5 kb,<sup>25</sup> representative of the carboxy-terminus of the NFKB2 gene (exons 22 through 24 and 3' untranslated region). The germline regions of chromosomes 10 and 7, involved in recombination with the NFKB2 gene in cases LB40 and EB308 and case LB363, respectively, were isolated using specific probes for chromosomes 10 and 7 (Figs 1 and 2) from a genomic library constructed by cloning partially *Sau*3A-digested DNA from human placenta in the  $\lambda$ EMBL3 phage vector. Probes were <sup>32</sup>P-labeled by the random priming method.<sup>26</sup> Screening was performed by plaque hybridization. Isolated plaques were grown and phage DNA obtained according to established procedures.<sup>27</sup> Inserts were analyzed by restriction enzyme mapping and subcloned into plasmid vector pGEM3 (Promega, Madison, WI) for further analysis.

**DNA sequencing.** DNA sequence analysis was performed on restriction fragments cloned into pGEM3 plasmid (Promega) by dideoxy chain-termination analysis using the Sequenase sequencing kit (USB, Cleveland, OH). When needed, nested deletion fragments were created by EXO-Mung digestion (Stratagene).

**Southern blot analysis.** Ten micrograms of genomic DNA was digested with the appropriate restriction enzyme, electrophorized in a 0.7% agarose gel, and then denatured, neutralized, and transferred to nylon filters (Amersham International, Amersham, UK). The filters were hybridized to probes <sup>32</sup>P-labeled by the random priming method<sup>26</sup> according to the manufacturer's specifications, washed in 0.5 × SSC (NaCl/Na citrate)/1% sodium dodecyl sulfate (SDS) for 1 hour at 60°C, and then autoradiographed using intensifying screen at -80°C.

**Somatic cell hybrids.** The somatic cell hybrids were obtained as already described.<sup>28</sup> Human lymphocytes or human fibroblast cells were fused with HPRT-CHO cell lines YH-21 or RJK88 following a published protocol.<sup>29</sup> The hybrid clones and subclones were characterized for their human chromosome content by cytogenetic analysis based on QM-banding and fluorescent in situ hybridization (FISH) experiments on normal human metaphases of Alu-polymerase chain reaction (PCR) products from each hybrid.

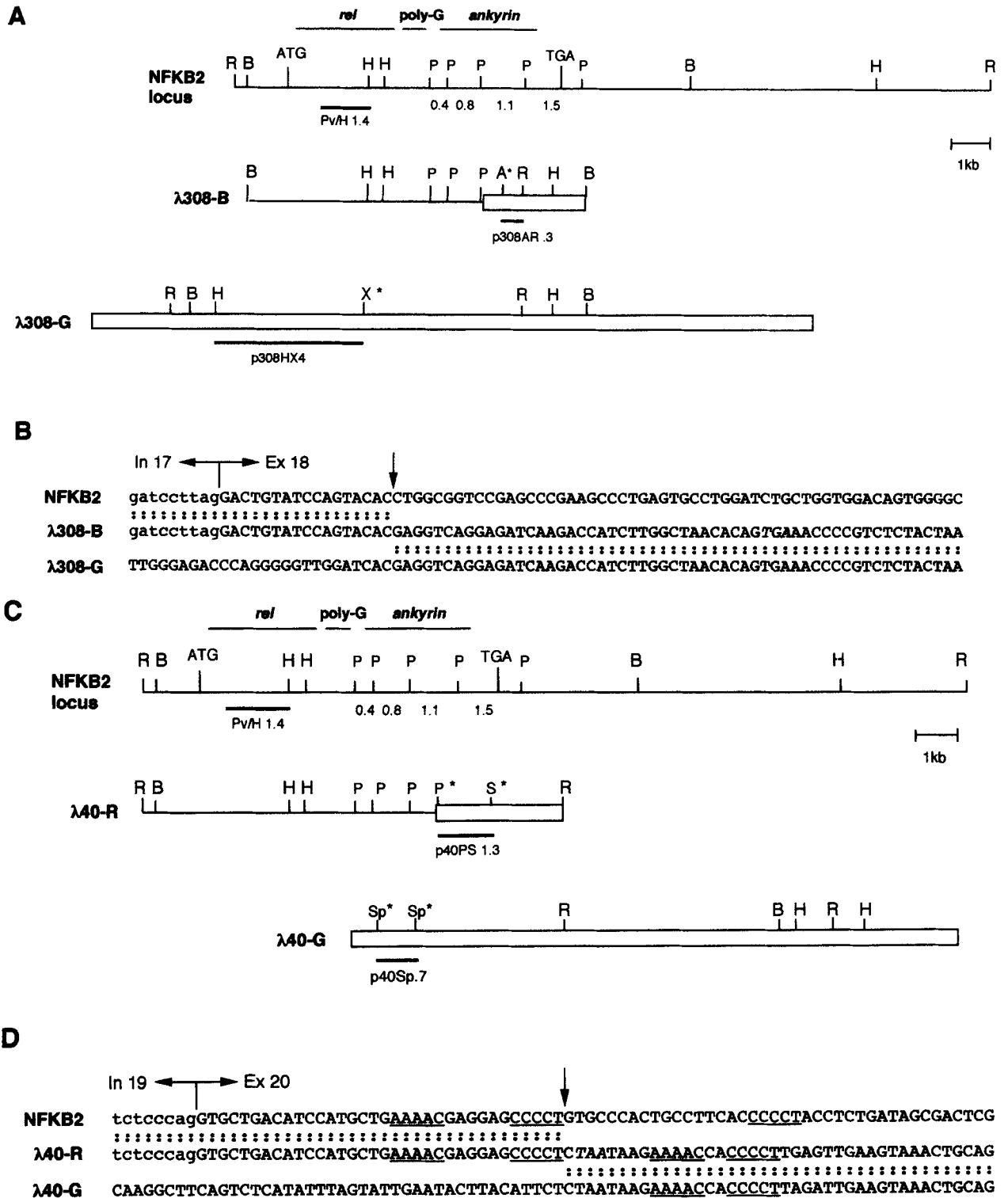
**PCR amplification of human/hamster somatic cell hybrids.** The DNAs (0.5  $\mu$ g each) from somatic cell hybrids were amplified in 50- $\mu$ L reactions containing 200  $\mu$ mol/L of each dNTP, 50 mmol/L KCl, 10 mmol/L TRIS (pH 8.3), 1.5 mmol/L MgCl<sub>2</sub>, 0.01% (wt/vol) gelatin, 2.5 U *Taq* polymerase (Boehringer, Mannheim, Germany), and 20 pmol/L of each specific primer. The following primers were used: LB40-5, 5'-TCACAGATGAGTTATCTAAG-3', and LB40-3, 5'AATAATTCTATCAGAGTGGA-3' (amplified fragment length, 264 bp); EB308-3, 5'-ACTCAGCATCTCTCATGAAG-3', and EB308-3, 5'-TGTGCTATGAGCAACTCTGT-3' (amplified fragment length, 275 bp); and LB363-5, 5'-GTCCAGGTTTCAGTGTGT-3', and LB363-3, 5'-AGCTGAGTCACTACATGG3' (amplified fragment length, 276 bp). Amplification reactions (30 cycles) were performed using a Perkin Elmer-Cetus (Norwalk, CT) DNA thermal cycler.

**Fibroblast culture.** Fibroblast cultures from a normal donor were starved for 3 days in medium containing 0.1% fetal calf serum to minimize the number of G2 cells.

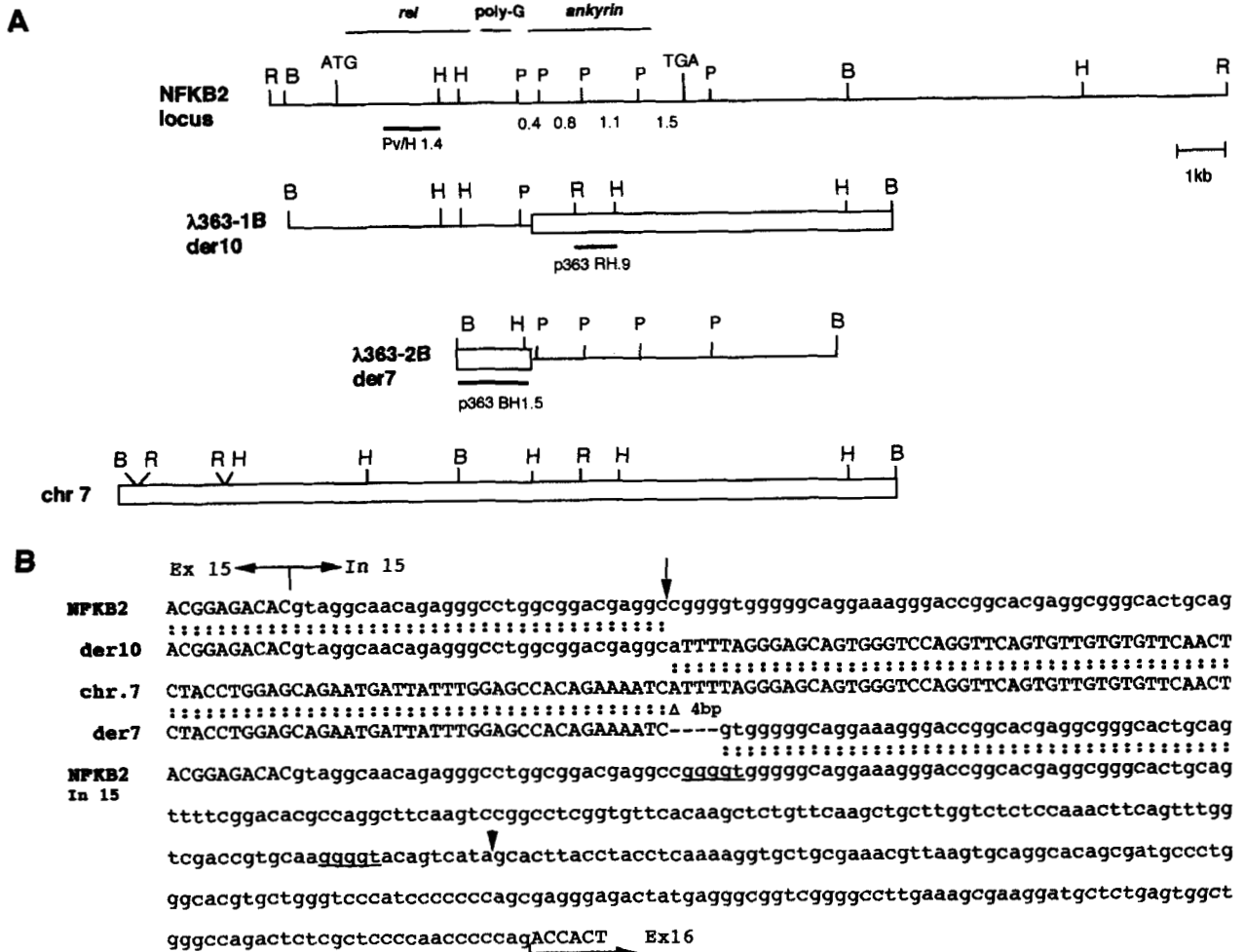
**FISH.** Human metaphase spreads were obtained from phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes from a human donor. Chromosome preparations were hybridized in situ with probes labeled with biotin or digoxigenin by nick translation, essentially as described,<sup>30</sup> although with minor modifications.<sup>31</sup> Chromosome identification was obtained by simultaneous DAPI staining that produces a Q-banding pattern. Digital images were obtained using a Zeiss Axioplan epifluorescence microscope (Zeiss, Oberkochen, Germany) equipped with a cooled CCD camera (Photometrics, Tucson, AZ). Fluorescein isothiocyanate (FITC), rhodamine, and DAPI fluorescence, detected using Pinkel #1 specific filter set combinations (Chroma Technology, Brattleboro, VT), were recorded separately as gray scale images. The filter set used allows DAPI, FITC, and rhodamine signals to be captured without any image shifting. Pseudocoloring and the merging of images were performed using GeneJoin software (developed by T. Rand in the laboratory of Dr D.C. Ward, Yale University, New Haven, CT); the same software package was used to measure the probe distances in interphase nuclei.

**cDNA amplification.** First-strand cDNA was synthesized in 20- $\mu$ L reactions containing 1  $\mu$ g total RNA extracted from cases LB40 and EB308, 1 mmol/L of each dNTP, 0.1 mmol/L dithiothreitol (DTT), 5× reverse-transcription buffer, 1 U RNasin (Promega), 200 U Super-Script reverse transcriptase (GIBCO-BRL, Gaithersburg, MD), and 20 pmol/L of primer specific for case LB40 (5'-TCAACTCAAGGGGTGTT-3') or EB308 (5'-TTCACGTGTGTTAGCCAAAG-3'). The reaction mixes were incubated at 37°C for 1 hour and stored at -20°C. PCR amplifications were performed by diluting 10  $\mu$ L of first-strand cDNA from each individual case into 50- $\mu$ L mixtures as described above. The 3' primer used in the PCR amplifications for each individual case was the same as that used in reverse transcription; the 5' primer specific for the NFKB2 cDNA (5'-TCAGAGTGAGCTCCTG-3' positions 1819 to 1835)<sup>6</sup> was the same in both cases. Amplification reactions were performed as described above in the following conditions: denaturing at 94°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The individual PCR products were cloned in the plasmid vector pCR II (Invitrogen, San Diego, CA) and sequenced by dideoxy chain-termination reaction analysis using the Sequenase sequencing kit (USB).

**Construction of expression vectors containing NFKB2-truncated cDNA.** To construct the NFKB2-truncated cDNAs specific for cases EB308 and LB40, the PCR fusion products cloned into the pCR II vector were released with *Sac*I (NFKB2 site) and *Eco*RI (polylinker site) restriction enzymes and ligated into the *Sac*I (NFKB2 cDNA site; position 1827) and *Eco*RI (polylinker site) of the full-length normal NFKB2 cDNA cloned in pGEM3 vector.<sup>6</sup>



**Fig 1.** Molecular cloning of rearranged NFkB2 genes from cases EB308 and LB40. (A and C) Schematic representations of the cloned rearranged NFkB2 genes ( $\lambda$ 308-B and  $\lambda$ 40-R clones) and their respective germ line counterparts (NFkB2 locus and  $\lambda$ 308 and  $\lambda$ 40G, respectively). (—) NFkB2 locus; (□) novel 10q24 regions from cases EB308 and LB40. The approximate locations of the start (ATG) and stop (TGA) codons of the NFkB2 gene are shown. The probes used for molecular cloning and Southern blot analysis are indicated below the diagrams. R, *EcoRI*; B, *BamHI*; H, *HindIII*; P, *Pst I*; A, *Acc I*; X, *Xho I*; S, *Sac I*; Sp, *Sph I*. \*Only sites delineating probes are shown. The respective location of the *rel* domain, poly-G tract and *ankyrin* domain of the NFkB2 gene within the restriction map is indicated. (B and D) Nucleotide sequence analysis of breakpoint regions and their alignment to corresponding germline regions. The sequences were obtained by analyzing both DNA strands and are derived from 401 bp (EB308) and 412 bp (LB40) across the respective breakpoints. The exon sequences of the NFkB2 gene are shown in capital letters, while the intron sequences are shown with small caps. Arrows indicate the break site locations. The putative new stop codons are shown in italics. The direct repeats across the breakpoint in case LB40 and in the respective germline counterparts are underlined.



**Fig 2. Molecular cloning of the chromosomal breakpoint from case LB363. (A) Schematic representation of the cloned reciprocal breakpoint regions (der 10 and der 7) and their respective germline counterparts (NFKB2 locus/chr 10 and chr 7). (—) Chromosome 10 regions; (□) chromosome 7 regions. The approximate locations of the start (ATG) and stop (TGA) codons of the NFKB2 gene are shown. The probes used for molecular cloning and Southern blot analysis are indicated below the diagrams. R, *EcoRI*; B, *BamHI*; H, *HindIII*; P, *PstI*. (B) Nucleotide sequence analysis of breakpoint regions and their alignment to the corresponding germline regions. The sequences were obtained by analysis of both DNA strands and are derived from 378 bp across the breakpoint. The exon sequences of the NFKB2 gene are shown in capital letters; the intron sequences are shown with small caps. The 4-bp deletion from intron 15 of the NFKB2 gene on der 7 is shown. Arrow indicates the breakpoint site. Arrowhead indicates the breakpoint site within intron 15 in case RC685.<sup>9</sup> The pentameric sequence GGGGT is underlined.**

The truncated versions of the NFKB2 cDNAs were released with *EcoRI* digestion and cloned into the *EcoRI* site of the expression vector pMT2T.<sup>13</sup> The fidelity of these two clones to the original NFKB2-truncated cDNA from cases EB308 and LB40 was confirmed by nucleotide sequencing.

**Indirect immunofluorescence staining.** HeLa cells ( $3 \times 10^5$ ) were attached to a microscope slide 16 hours before the experiment. Transfections with 1 pmol/L of a different plasmid were performed using the CaPO<sub>4</sub> precipitation procedure.<sup>27</sup> The slides were further fixed in phosphate-buffered saline (PBS) containing 3% paraformaldehyde for 30 minutes, washed in PBS, and permeabilized in PBS containing 0.2 Triton X-100. The slides were blocked with 3% bovine serum albumin (BSA)-PBS for 4 hours at room temperature, followed by incubation with polyclonal antiserum 8892 (1:2,000 dilution) directed against the N-terminus of the NF- $\kappa$ B1p100<sup>13</sup> in 3% BSA-PBS for 12 hours at 4°C. FITC-conjugated swine IgG directed against rabbit IgG in 3% BSA-PBS was subsequently added

to the slides and incubated for 30 minutes at room temperature. The slides were intensively washed and mounted in 50% glycerol-PBS. Photographs were taken using a Leitz Dialux 20 microscope (Leitz, Wetzlar, Germany).

**RESULTS**

**Molecular cloning of rearranged NFKB2 genes.** In a previous study, we found rearrangements of the NFKB2/*lyt-10* gene in five of 228 cases representative of the different types of lymphoid malignancies: one case of B-cell chronic lymphocytic leukemia (B-CLL), one case of multiple myeloma (MM), one case of cutaneous B-cell lymphoma (CBCL), and two cases of cutaneous T-cell lymphomas (CTCL).<sup>25</sup> Southern blot analysis using genomic probes specific for different regions of the NFKB2 locus suggested

that the rearrangements involved the carboxy-terminus of the gene leading to the partial or total deletion of the *ankyrin* domain.

The origin of the NFKB2 rearrangements was investigated by isolating the rearranged NFKB2 restriction fragments from three of these five cases. No cytogenetic data were available. DNA from B-CLL case LB40 was digested to completion using the *EcoRI* restriction enzyme, whereas DNAs from MM case LB363 and CTCL case EB308 were digested to completion using the *BamHI* enzyme. Recombinant phage libraries were constructed using the gel-purified fractions containing the rearranged NFKB2 restriction fragments detected by Southern blot analysis.

Libraries generated from cases EB308 and LB40 were screened with the 5' genomic NFKB2 probe, Pv/H 1.4 kb (see scheme in Fig 1). Recombinant clones containing the rearranged NFKB2 fragment from each case were isolated and compared with the germline NFKB2 locus as previously reported<sup>25</sup> (Fig 1A and C). Restriction map analysis and hybridization with different probes from this locus showed that, in both the  $\lambda$ 308-B and the  $\lambda$ 40-R clones, the rearrangement occurred within the NFKB2 *PstI* 1.1-kb fragment. This genomic fragment includes exons 18 through 21 and the 5' border of exon 22, which code for *ankyrin* repeats IV through VII. As shown in Fig 1B and D, comparative sequence analysis of the regions encompassing the NFKB2 rearrangement in cases EB308 and LB40, with the normal NFKB2 sequence, showed that the break in clone  $\lambda$ 308-B occurred within exon 18, 18 bp downstream of its splice acceptor site; in clone  $\lambda$ 40-R, it occurred within exon 20, 34 bp downstream of its splice acceptor site.<sup>25</sup> Thus, in case EB308, the break was within the sequences coding for the fourth *ankyrin* repeat, at position 1888 of the normal NFKB2 cDNA.<sup>6</sup> As a consequence, part of the fourth *ankyrin* and all of the remaining carboxy-terminal portion were lost. In case LB40, the break occurred within the coding region between the sixth and the last (seventh) *ankyrin* repeat, at position 2178 of the normal NFKB2 cDNA, resulting in the loss of the seventh *ankyrin* and the remaining carboxy-terminal region. PCR analysis of a panel of somatic hamster-human hybrids using a specific pair of primers (see Materials and Methods) showed that the unidentified genomic regions linked to the NFKB2 sequences in both clones  $\lambda$ 308-B and  $\lambda$ 40-R originated from chromosome 10 (data not shown; see below). A data base homology search of the novel 3' sequences showed the presence of an *Alu* repetitive sequence immediately downstream of the junction site in clone  $\lambda$ 308-B; in clone  $\lambda$ 40-R, no homology with any already known sequences was found. As shown in Fig 1D, the break in NFKB2 in case LB40 laid between two 5-bp direct repeats; in addition, it involved a homologous stretch of nucleotides at both sides leading to the presence of direct repeats across the junction site in clone  $\lambda$ 40-R. Probes derived from the novel sequences in clones  $\lambda$ 308-B and  $\lambda$ 40-R were used to isolate the corresponding germline genomic regions by screening a human placental genomic library (see restriction enzyme maps of clones  $\lambda$ 308-G and  $\lambda$ 40-G and their normal nucleotide sequences encompassing the corresponding break sites in Fig 1). Hybridization of these two normal genomic

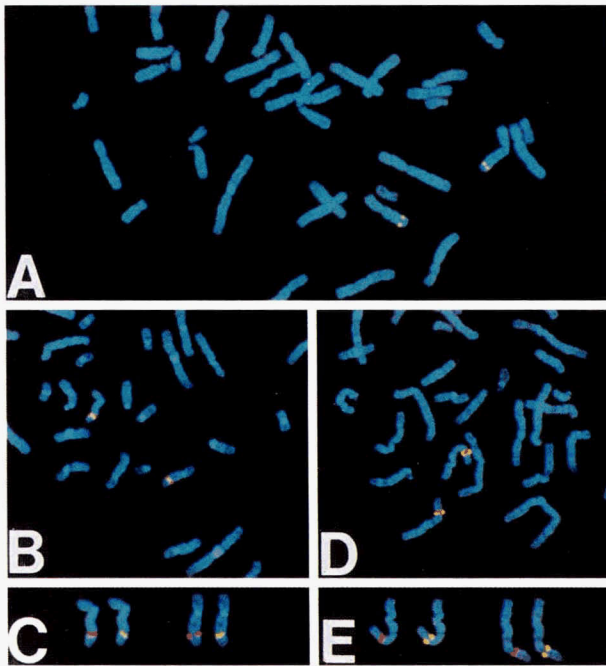
clones to probes derived from the 5' and 3' region of the NFKB2 locus<sup>24</sup> did not show any homology.

Regarding case LB363, hybridization of *BamHI* digests with probes specific for the 5' and 3' portion of the NFKB2 gene the Pv/H 1.4 kb and the *PstI* 1.5 kb, respectively) showed the presence of two differently rearranged NFKB2 fragments compatible with the occurrence of a reciprocal recombination event, such as a chromosomal translocation or DNA inversion.<sup>25</sup> These two rearranged fragments were cloned, and restriction enzyme mapping and hybridization analysis indicated that the divergence from the normal NFKB2 locus occurred within the germline *PstI* 0.4-kb fragment (Fig 2A). In particular, clone  $\lambda$ 363-1B contained the amino-terminus of the gene (*rel* domain and poly-G), whereas clone  $\lambda$ 363-2B contained the carboxy-terminus (*ankyrin* domain). Sequencing analysis of the breakpoints on the NFKB2 gene showed that they both occurred within intron 15 of the gene (Fig 2B). In particular, the breakpoint in clone  $\lambda$ 363-1B was located 30 bp downstream from the splice donor site of exon 15 and 156 bp upstream of the breakpoint site on the NFKB2 gene of the previously reported case.<sup>6</sup> In both clones, the NFKB2 regions were linked to novel sequences that originated from chromosome 7 as demonstrated by PCR analysis of a representative panel of somatic cell hybrids using a specific pair of primers (see Materials and Methods; data not shown). Thus, in case LB363, the entire carboxy-terminus portion of the rearranged NFKB2 allele containing the *ankyrin* domain was lost and translocated to a region of chromosome 7. The germline counterpart of this region was isolated by screening a human placental DNA library with probes 363RH and 363BH obtained from the novel regions linked to NFKB2 sequences in clones  $\lambda$ 363-1B and  $\lambda$ 363-2B, respectively (see restriction enzyme map in Fig 2A). Comparative analysis of the nucleotide sequences across the breakpoints on clones  $\lambda$ 363-1B and  $\lambda$ 363-2B, with the normal NFKB2 and chromosome 7 sequences (Fig 2B), indicated that the chromosomal translocation represented a fairly precise reciprocal recombination event, since only a 4-bp deletion from the intron 15 sequence of the NFKB2 gene in clone  $\lambda$ 363-2B was observed. Interestingly, the break at exon 15 involved the pentameric sequence GGGGT, which is characteristic of the switch region of the IgH locus.

Probes from each of the three germline regions failed to detect any transcripts in Northern blot analysis of a panel of total RNAs obtained from several human cell lines representative of different tissues (data not shown), thus suggesting the absence of transcribed genes in the proximity of the breakpoints. Finally, preliminary Southern blot analysis using the same probes did not show any rearrangements in 100 cases of lymphoid tumors, including B-CLL, CTCL, and MM.

*Chromosome mapping of the genomic regions involved in the rearrangements of the NFKB2 gene.* To determine the chromosome origin of the novel genomic regions juxtaposed to the NFKB2 sequences in the rearranged cases, we first performed PCR amplification of a representative panel of human-hamster somatic hybrid DNAs<sup>28</sup> using primers homologous to sequences derived from these regions. This



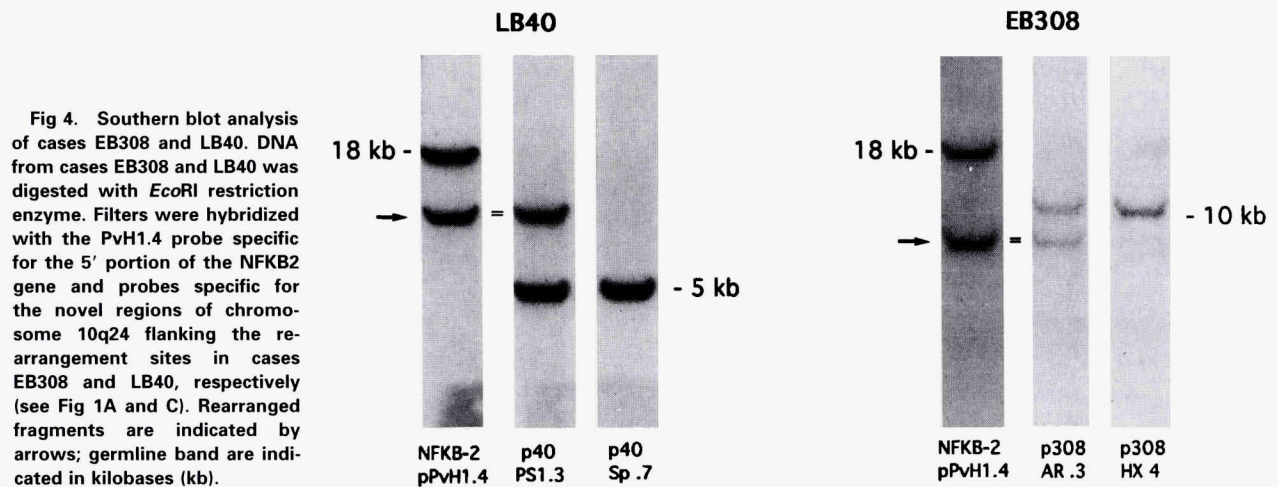


**Fig 3. Chromosomal mapping of the novel genomic regions rearranged with the NFKB2 gene.** The FISH experiments were performed on normal human metaphases. (A) Hybridization signals of phage 363-G are located at 7q34 (yellow signals). (B and C) Biotinylated NFKB2 cDNA (yellow signals) was cohybridized with phage insert 308-G labeled with digoxigen (red signals). (D and E) Biotinylated NFKB2 cDNA (yellow signals) was cohybridized with phage insert 40-G labeled with digoxigen (red signals). In both cases, the two signals localized at 10q24 were not spatially resolvable. (C and E) The signals of the cohybridized probes are reported separately. In all of the experiments, biotinylated probes were detected with FITC-conjugated avidin; digoxigenin-labeled probes were detected by rhodamine-conjugated antidioxigenin antibodies.

analysis showed that, in both the EB308 and LB40 cases, the presence of the expected amplified fragments correlated with the presence of the human chromosome 10; in case LB363, only hybrids containing the human chromosome 7

showed the specific amplified fragment after PCR (data not shown). To confirm these data and map these loci to specific chromosomal bands, phage inserts representative of the normal counterpart of the genomic regions involved in these three rearrangements were used as probes in FISH experiments on metaphase spreads from mitogen-stimulated normal blood lymphocytes (Fig 3). In case LB363, the phage insert 363-G clearly hybridized to chromosome 7q34 (Fig 3A). For both EB308 and LB40, the corresponding phage inserts 308-G and 40-G were cohybridized in situ with the NFKB2 probe (NFKB2 cDNA).<sup>6</sup> In both experiments (Fig 3B and C; Fig 3D and E), the two signals were found to map in the 10q24 chromosomal region where the NFKB2 gene is located.<sup>6,32</sup> The two signals were not spatially resolvable on metaphase chromosomes, suggesting that their distance is less than the resolution limits of the technique (< 2 to 3 Mb). We therefore performed FISH experiments on the interphase nuclei of starved fibroblasts from a normal donor, an approach that makes it possible to determine distances in the 50-kb to 1-Mb range.<sup>33</sup> Forty independent measurements were performed for each pair of signals (NFKB2/308-G and NFKB2/40-G) with the following mean results: NFKB2/308-G, 197 nm; NFKB2/40-G, 686 nm. According to Lawrence et al,<sup>33</sup> the distance NFKB2/308-G should be less than 100 kb, while NFKB2/40-G is approximately 400 kb.

*Rearrangements in cases EB308 and LB40 represent chromosome 10q24 internal deletions.* The rearrangements in cases EB308 and LB40 may have arisen by different mechanisms, such as local DNA inversion or deletion. In the case of local DNA inversion, molecular analysis should identify rearranged fragments due to the fusion event reciprocal to that generating the carboxy-truncated forms of the NFKB2 gene, probably without the loss of genetic material. Our previous Southern blot analysis of these two cases using probes specific for the 3' regions of the NFKB2 locus showed the absence of any rearranged fragments.<sup>25</sup> This finding suggested that the NFKB2 rearrangements occurred with the loss of genetic material, which was confirmed using probes derived from the λ308-G and λ40-G phage inserts

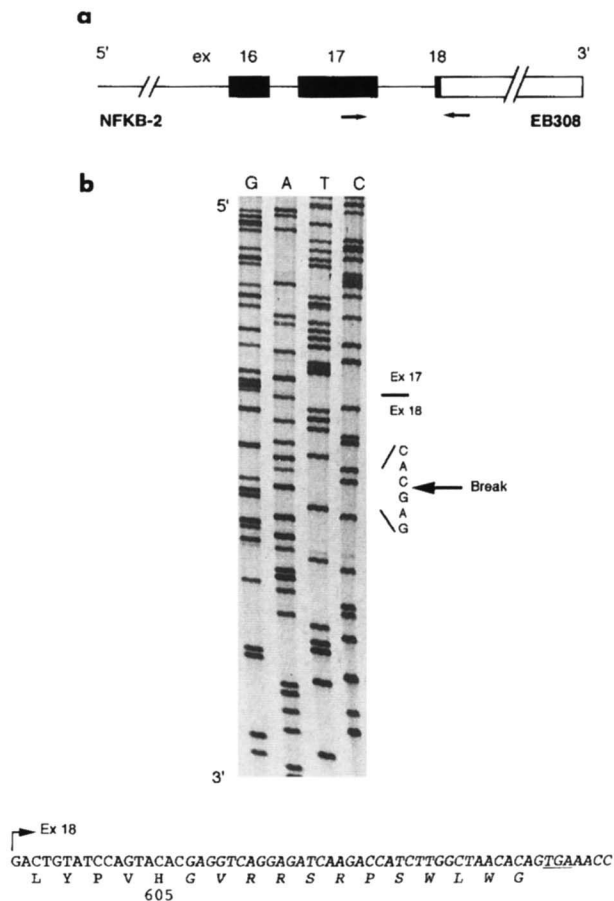


**Fig 4. Southern blot analysis of cases EB308 and LB40.** DNA from cases EB308 and LB40 was digested with *EcoRI* restriction enzyme. Filters were hybridized with the Pvh1.4 probe specific for the 5' portion of the NFKB2 gene and probes specific for the novel regions of chromosome 10q24 flanking the rearrangement sites in cases EB308 and LB40, respectively (see Fig 1A and C). Rearranged fragments are indicated by arrows; germline band are indicated in kilobases (kb).

located upstream of the breakpoint sites. As shown in Fig 4, probes 308HX4 and 40Sp.7 (see schemes in Fig 1A and C) did not detect rearranged fragments in DNA derived from cases EB308 and LB40, respectively. As expected, probes 40PS1.3 and 308AR.3 detected the same rearranged fragments that were identified in these two cases by the PvH1.4 NFKB2 probe (Fig 4). These results are thus consistent with a model in which the NFKB2 rearrangements are generated by DNA deletion and a joining of the sequences between the two recombining regions.

**Identification of truncated NFKB2 transcripts in vivo.** The presence of abnormal NFKB2 transcripts in rearranged cases was only investigated in cases EB308 and LB40, for which total RNA was available. However, because of the small quantity and degraded status of the RNA in both cases, Northern blot was not possible. We therefore used a PCR-based strategy to detect abnormal NFKB2 mRNAs in these two cases. As described above, the rearrangement in both cases had fused sequences within different exons of the NFKB2 gene to a novel genomic region in chromosome 10q24. This suggested that if the NFKB2 genes were transcribed from the rearranged allele, the transcription might continue in the novel region ending at a putative stop codon sequence, generating a carboxy-terminal truncated version of the NFKB2 mRNA. Sequencing analysis showed the presence of a putative stop codon 36 nucleotides downstream from the breakpoint in case EB308, and immediately after the breakpoint in case LB40 (Fig 1A and C). We used oligonucleotide primers homologous to the sequences across these putative stop codons and at the 3' border of exon 17 of the NFKB2 gene to amplify the cDNAs reverse-transcribed from the EB308 and LB40 RNAs. In both cases, a specific fragment of the expected size (110 bp for case EB308, and 388 bp for case LB40), presumably spanning the junction of the NFKB2-EB308 or NFKB2-LB40 fusion transcripts, was obtained from the respective cDNA, but not from the cDNAs of a number of cell lines without NFKB2 rearrangements (data not shown). Sequencing analysis of PCR products confirmed the nature of these amplified fragments (Figs 5 and 6), and indicated the presence of a truncated version of NFKB2 mRNA in tumoral cells from cases EB308 and LB40. In case EB308, analysis of the predicted protein product indicated a 3' truncated NFKB2 protein of 617 amino acids, lacking a part of the fourth *ankyrin* and all of the fifth to seventh *ankyrin* repeats, and containing a tail of 12 amino acids at its 3' terminus, derived from the novel sequences of chromosome 10q24. In case LB40, analysis indicated a 3' truncated NFKB2 protein of 702 amino acids, lacking the last *ankyrin* repeat (see scheme in Fig 7).

**Subcellular localization of NFKB2-truncated proteins.** To determine the subcellular localization of the putative truncated NFKB2 proteins originated from the rearrangement in cases EB308 and LB40, we constructed expression vectors containing the NFKB2/EB308 and NFKB2/LB40 cDNA fusion sequences. HeLa cells were transfected with plasmid constructs expressing the two putative NFKB2-truncated proteins, as well as those expressing NF- $\kappa$ B2p100 and p52, and analyzed by indirect immunofluorescence (Fig 8). As expected, NF- $\kappa$ B2p52 was localized within the nucleus,



**Fig 5. Identification of the NFKB2-truncated transcript in case EB308.** (a) Schematic representation of the NFKB2 rearrangement in case EB308. (—) Introns of the NFKB2 gene; (■) exons. (□) Novel region from chromosome 10q24 involved in the rearrangement. The position of the 5' and 3' primers used in the RT/PCR analysis is shown below the map (see Materials and Methods). (b) Sequencing of the PCR-amplified NFKB2-truncated cDNA from EB308 cells (see Materials and Methods). The arrow indicates the junction between NFKB2 and the novel chromosome 10q24 region in case EB308 as found in the rearranged genomic clone (see nucleotide sequence in Fig 1B); the splicing junction between exon 17 and exon 18 of NFKB2 is indicated by a dash. The rearranged NFKB2 sequence is reported below the sequence ladder; the sequence from chromosome 10q24 rearranged with the NFKB2 gene is represented by italic letters, as well as the novel putative 12 amino acids predicted from the in-frame reading of the NFKB2 sequence. The putative stop codon is underlined. The number below the amino acid sequence refers to that previously reported.<sup>6</sup>

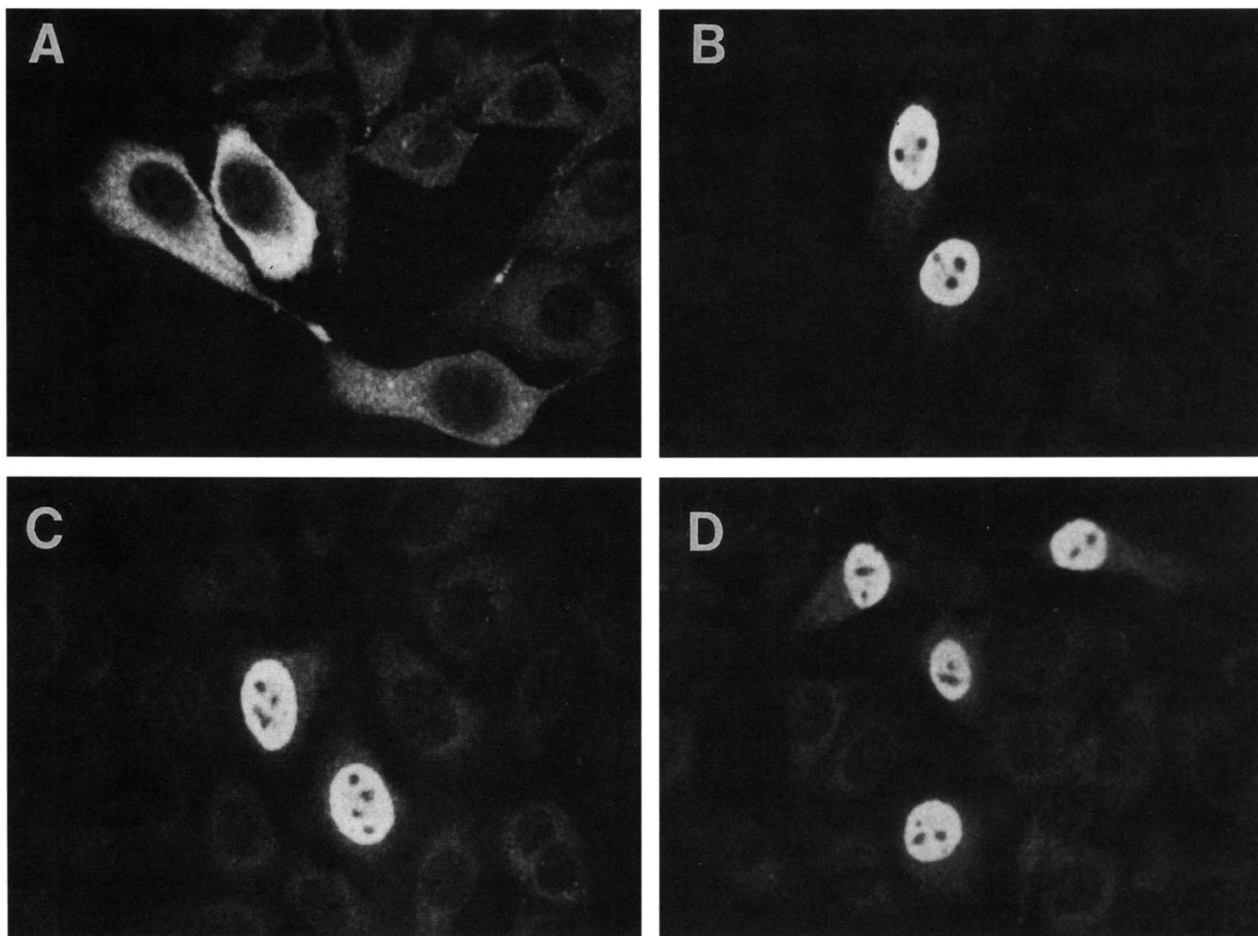
while NF- $\kappa$ B2p100 was exclusively cytoplasmic. Truncated NFKB2 proteins from cases EB308 and LB40 were found to be localized in the nucleus, thus suggesting their constitutive activation in vivo.

## DISCUSSION

The NFKB2/*lyt-10* gene is a member of the NF- $\kappa$ B gene family, which we have previously identified as a candidate proto-oncogene involved in a chromosomal translocation t(10;14)(q24;q32) associated with a case of B-cell







**Fig 8.** Subcellular localization of the normal and rearranged NF $\kappa$ B2 proteins as described in Materials and Methods, HeLa cells were separately transfected with NF $\kappa$ B2p100 (A), NF $\kappa$ B2p52 (B), NF $\kappa$ B2/EB308 (C), and NF $\kappa$ B2/LB40 (D), stained with anti-NF $\kappa$ B2p52 antibody<sup>13</sup> and fluorescein-conjugated secondary antibody, and examined under fluorescent microscopy.

cate that the NF $\kappa$ B2 gene may be structurally altered by heterogeneous chromosomal aberrations, such as chromosomal translocation with different chromosomal translocation partners or, more frequently, small internal deletions.

The data presented here suggest that the abnormal recombination of the NF $\kappa$ B2 gene may be mediated by different DNA recombination mechanisms. In case LB363, the breakpoint on chromosome 10q24 was localized within intron 15 of the NF $\kappa$ B2 gene. This was an interesting finding, since the breakpoint on the NF $\kappa$ B2 gene of the first case reported<sup>6</sup> occurred within the same intron, 156 nucleotides downstream from the breakpoint found in case LB363. This suggests the presence of sequences in this intron that may be prone to recombination events. Nucleotide sequence analysis of intron 15 of the NF $\kappa$ B2 gene shows that the breakpoints in case LB363, as well as in the previous B-NHL case,<sup>6</sup> occurred closely to or within (case LB363) the pentameric sequence GGGGT, which is characteristic of all the switch regions of the IgH gene and is thought to be involved in the switching mechanisms.<sup>39</sup> These findings suggest that rearrangement of the NF $\kappa$ B2 gene in these two cases could represent mistakes in the normal immunoglobu-

lin switching mechanisms, which are particularly active in mature B cells. In the remaining two cases, the break sites on the NF $\kappa$ B2 locus involved two different exons. The recombination event in case EB308 had joined exon 18 of the NF $\kappa$ B2 gene with an *Alu* repetitive sequence. *Alu* sequences are considered to be a type of transposable element, and therefore capable of mediating DNA recombination.<sup>40</sup> In case LB40, the break on exon 20 of NF $\kappa$ B2 occurred between two direct repeats. The presence of repeats near breakpoint junctions is a feature that has been observed in several types of chromosomal translocation,<sup>41,42</sup> as well as in the deletion and insertion of DNA sequences.<sup>43</sup>

**Implications of NF $\kappa$ B2 alterations in tumorigenesis.** Despite the heterogeneous nature of the mechanisms involved in the abnormal recombination of the NF $\kappa$ B2 gene, the common denominator of these lesions appears to be the generation of NF $\kappa$ B2 proteins retaining the DNA binding *rel* domain, lacking variable portions of the carboxy-terminal *ankyrin* domain and showing an exclusively nuclear localization. With regard to NF $\kappa$ B1 and NF $\kappa$ B2 genes, it is likely that their *ankyrin* domains play an important physiologic role in the regulation of transcriptional activity by contributing to

the cytoplasmic retention of their unprocessed proteins, p105 and p100, respectively. It has been suggested that the mechanism by which this domain contributes to cytoplasmic sequestration may involve the intramolecular masking of the nuclear localization signal (NLS) of the NF- $\kappa$ B proteins. The NLS is a short basic region located immediately carboxy-terminal to the *rel* homology domain and it is thought to allow the nuclear translocation of NF- $\kappa$ B complexes.<sup>10,44</sup> A similar mechanism involving the *ankyrin* domain has been proposed for the sequestering of NF- $\kappa$ B proteins in the cytoplasm by I $\kappa$ B proteins.<sup>45</sup> With regard to NF- $\kappa$ B1p105 precursor, deletion analyses have led to the identification of sequences within its *ankyrin* domain that are likely to be responsible for its cytoplasmic retention by masking the NLS, as well as the lack of DNA binding activity and the control of the extent of its proteolytic processing. In particular, Henkel et al<sup>44</sup> have demonstrated that 3' deletion of the NF- $\kappa$ B1p105, including the seventh *ankyrin* and the carboxy-terminus, led to its nuclear localization, while other investigators<sup>10</sup> have shown that the removal of the sixth *ankyrin* and, to a lesser extent, the 3' adjacent acidic region was necessary to obtain a predominant nuclear localization of the NFKB1 protein. However, it has been suggested that the integrity of the carboxy-terminal region of p105 is probably required to make a correct and efficient intramolecular interaction.<sup>10</sup> Given the homology between the NF- $\kappa$ B1p105 and NF- $\kappa$ B2p100, the structural and functional characteristics of the tumor-associated truncated NFKB2 proteins described here (in particular case LB40) suggest that the acidic domain located between the last two *ankyrin* repeats and/or the seventh *ankyrin* repeat may be involved in the cytoplasmic localization of the p100 precursor. It is interesting to point out that preliminary results obtained by transfection assays of different carboxy-truncated forms of NF- $\kappa$ B2p100 generated in vitro seem to suggest that sequences involved in the cytoplasmic retention of p100 may be located 3' to the *ankyrin* domain (L. Lombardi and A. Neri, unpublished data).

The exclusively nuclear localization of the tumor-associated putative truncated NFKB2 proteins observed in our transfection experiments may suggest a constitutive nuclear localization of these proteins in vivo. In the nucleus, normal NF- $\kappa$ B2p52 has been shown to act as a transcriptional activator by formation of heterodimers with p65 or other *rel* molecules, or as a transcriptional repressor by formation of p52 homodimers.<sup>13</sup> Furthermore, it has been demonstrated that p52 homodimers may associate with *bcl-3* forming a potent transactivating complex.<sup>22</sup> In addition, p52 appears to regulate the transcription of its own gene negatively (L. Lombardi and A. Neri, manuscript in preparation). Based on these experimental evidences, it may be suggested that abnormal constitutively nuclear NFKB2 proteins may alter NF- $\kappa$ B activity in vivo both quantitatively (high levels of nuclear NFKB2 protein) and qualitatively (abnormal homo- and hetero-dimerization, DNA binding).

Transcription factors represent a frequent target for oncogenic activation.<sup>46</sup> In the last few years, several novel genes acting as transcription factors have been found to be altered by chromosomal translocations in hematologic malignancies,

suggesting an important role of transcriptional deregulation in these neoplasias.<sup>47-49</sup> In addition to NFKB2/*lyt-10* gene, other genes of the NF- $\kappa$ B and I $\kappa$ B families have been found altered in lymphoid malignancies. Rearrangements or amplifications of the *c-rel* oncogene have been reported in human B-cell lymphomas,<sup>50</sup> and the *bcl-3* gene has been found to be involved in some B-CLL cases carrying t(14;19) chromosomal translocation.<sup>21</sup> Although the frequency of each of these alterations is relatively low, taken together these findings suggest that the alteration of the NF- $\kappa$ B transcription system may play a role in tumorigenesis. NF- $\kappa$ B genes normally mediate the expression of factors involved in the control of lymphoid growth and differentiation, such as cytokines or cytokine receptors.<sup>1,2</sup> It is conceivable that impairment of this regulation as a result of abnormal NF- $\kappa$ B activity may be involved in lymphomagenesis.

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