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Detection of Abundantly Transcribed Genes and Gene Translocation in Human Immunodeficiency Virus–Associated Non-Hodgkin's Lymphoma[1]

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Abstract

Several novel, differentially transcribed genes were identified in one centroblastic and one immunoblastic HIV-associated B-cell non-Hodgkin's lymphoma (B-NHL) by subtractive cloning. In both lymphomas, we detected an upregulated transcription of several mitochondrial genes. In the centroblastic B-NHL, we found a high level transcription of nuclear genes including the interferon-inducible gene (*INF-ind*), the immunoglobulin light chain gene (*IgL*), the *set* oncogene, and several unknown genes. The data obtained on upregulated expression of the genes in human B-NHL of HIV-infected patients considerably overlap with those obtained earlier for the B-NHL of simian immunodeficiency virus–infected monkeys. In the centroblastic lymphoma, one transcript revealed a fusion of the 3′-untranslated region of the *set* gene and the C-terminal region of the *IgL* gene. This chimeric sequence was confirmed by a site-directed polymerase chain reaction performed with total cDNA and genomic DNA. The expected amplification product was obtained in both cases pointing to a genomic rearrangement. The *IgL*–*set* fusion sequence was not found in cDNA preparations and genomic DNA of the immunoblastic HIV-associated B-NHL. Further studies are necessary to determine whether these genes contribute to lymphoma development or can be used as therapeutic targets.
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Keywords: HIV, B-cell lymphoma, transcription, genomic rearrangement, subtractive cloning.

Introduction

Humans infected with human immunodeficiency virus (HIV) are predisposed to develop B-cell non-Hodgkin's lymphomas (B-NHL) with distinct histomorphological characteristics [1,2]. Studies on the mechanisms of lymphomagenesis have shown that several molecular alterations seem to be associated with tumor development. Different groups of genes may contribute to the chain of events that eventually lead to lymphomagenesis. These groups are comprised of oncogenes, tumor suppressor genes, genes encoding intratumoral cytokines, growth factors, growth factor receptors, transcription factors, and cell adhesion proteins [2–4]. Cellular genes playing a role in growth regulation, cell senescence, and proliferation such as *c-myc*, *bcl-1*, *bcl-2*, *bcl-6* and *p53* are essential for growth transformation of lymphoma cells. Malignization of cells during lymphomagenesis is also related to genetic lesions in tumor cell chromosomes, e.g., rearrangements and mutations of genes. Some of the alterations cause the formation of novel fused genes [5–8]. In addition, overexpression of some housekeeping genes takes place [9–11].

Viral cofactors of lymphomagenesis have also been postulated. Epstein-Barr virus (EBV) infection has long been associated with Burkitt's lymphoma. It is present in almost 100% of endemic cases and up to 30% in sporadic cases [12]. The prevalence of EBV genomes in tumor cells is about 30% in acquired immunodeficiency syndrome (AIDS)–associated NHLs [1–3]. The incidence of B-NHL is about 10% in HIV-infected patients. However, the role of this herpes virus as well as the immunodeficiency virus as cofactor or etiological agent in the lymphomagenesis is not clear. HIV-associated B-NHL shares some histological and molecular characteristics with spontaneous lymphomas. Basic differences with respect to gene expression were not detected. However, AIDS-associated B-NHL exhibits unique features that distinguish them significantly from NHL arising in individuals with iatrogenic, congenital, or non-HIV immunodeficiencies [13,14]. These findings strongly suggest the presence of unique mechanisms leading to AIDS-associated NHL.

Multiple factors presumably contribute to the development of the AIDS-associated NHL including chronic antigenic stimulation — a tendency towards chromosomal transloca-

Abbreviations: AIDS, acquired immunodeficiency syndrome; AS, ATP synthase; B-NHL, B-cell non-Hodgkin's lymphoma; COX, cytochrome *c* oxidase; CYTb, cytochrome *b*; IgL, immunoglobulin light chain; *INF-ind*, interferon-inducible gene; ND, NADH dehydrogenase; IL4R, interleukin 4 receptor; TAP2, ABC transporter protein 2 located in human MHC class II; PCR, polymerase chain reaction; SIV, simian immunodeficiency virus; EBV, Epstein-Barr virus; HIV, human immunodeficiency virus

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tions and gene products of HIV itself [2,3,15,16]. In particular, the *tat* gene of HIV-1 is reported to have oncogenic potential [15,16] and *tat* can enhance the migration of lymphoma cells and their adhesion to endothelial cells [17].

In order to clarify the mechanisms of lymphomagenesis, several new approaches have been recently proposed [18–21]. The methods allowed to get spectra of genes differently expressed in malignant cells, to more correctly characterize different types of lymphomas, and to reveal new diagnostic markers for them.

Our study aimed to identify genes that are differentially expressed or overexpressed in HIV-associated lymphoma by polymerase chain reaction (PCR)–based subtractive cloning. This kind of expression profiling extends our previous studies describing cytokine gene transcription patterns in HIV-associated human and simian immunodeficiency virus (SIV)–associated monkey lymphomas [4]. Besides, recently, we detected an upregulation of several nuclear and mitochondrial genes in SIV-associated B-cell monkey lymphomas [21]. Our experimental approach allowed us to detect genes which have not yet been thought to be upregulated in human AIDS-associated lymphomas. In addition, we detected for the first time a gene fusion between the *IgL* gene and the rarely described *set* gene.

Materials and Methods

Tumor Tissue

Biopsy specimens from lymphomas A and B both from HIV-1–infected AIDS patients (males, ages 43 and 36) were kindly provided by Prof. Dr. I. Schedel (Medical School, Hanover, Germany). Histological and virological characteristics of these tumors are summarized in Table 1. Material from lymphoma A was taken from the left tonsil. Specimens from lymphoma B were taken from the liver hilus. The latter patient was classified as WR-6 stage of AIDS. Both tumors were B-NHLs either of the centroblastic type (lymphoma A) or the immunoblastic type (lymphoma B). Cells from both tumors harbored EBV genomes, and EBER-1 as well as EBNA-2 mRNAs were present [22].

Table 1. Characteristics of Two AIDS-Associated B-NHLs.

Histological and Virological Characteristics	Lymphoma A	Lymphoma B
REAL classification	Diffuse large B cell	Diffuse large B cell
KIEL classification	Centroblastic	Immunoblastic
Ki 1	Positive	Positive
Tumor site	Extranodal tonsils	Extranodal lymph node
Stage of immunodeficiency	–	WR 6
EBV serology	Positive	Positive
EBNA-2	Positive*	Positive*
HIV-1 serology	Positive	Positive
p24	Positive	–

*Immunohistochemistry and/or mRNA *in situ*.
(–) No data available.

DNA and RNA Isolation

Cellular DNA was isolated from tissue biopsy specimens stored in liquid nitrogen. Tissues were dispersed and lysed in eight volumes of a buffer containing 0.5 M EDTA, pH 8.0, 0.4% sarcosyl [23]. DNA was extracted twice with phenol and precipitated with 96% ethanol. The DNA was stored in Tris–EDTA buffer at a concentration of 100 ng/μl.

Total cellular RNA was isolated from tissues dispersed in liquid nitrogen. It was isolated in the presence of 4 M guanidine isothiocyanate [23]. RNA was extracted twice with phenol and the RNA concentration was determined spectrophotometrically. The quality of the isolated RNA was confirmed by a horizontal agarose gel electrophoresis.

Subtractive Cloning

A PCR-based technique was performed. As tracer, we used RNA from lymphoma cells and as driver RNA from B lymphocytes of an uninfected human or from other types of lymphoma. A detailed protocol on how to generate cDNA libraries, isolation of lymphoma-specific cDNA by subtractive hybridization, and the differential screening was published [21,24,25]. In general, schematic representation of the approach is presented in Figure 1.

PCR Analyses of Total cDNA and Genomic DNA to Detect Fused Ig–set Sequences

The PCR analyses were performed with 500 ng of genomic DNA and 30 ng of cDNA. The reaction was performed in a 20-μl volume containing 10$\times$ PCR buffer (Promega), 0.3 mM of each dNTP (Fermentas), 10 pmol of each primer, and 0.2 μ of *Taq*I polymerase. The following primers were used: set1: 5′-AAACAAGAGAAAGTAGACAG-3′; set2: 5′-CCTGTGTAGTAGTGTATAG-3′; set3: 5′-TGTTGGTAAATGCTAACTGTCCA-3′; set4: 5′-CTTGGCATTAGAGCACCAGG-3′; set5: 5′-GGAGCTCAACTCCAACCACGACGGG-3′; set6: 5′-CCTCTCCTAACTCATCAGCACCTGC-3′; Ig1: 5′-AATGAGGATATTTATTGGGG-3′; Ig2: 5′-TTTCATGAGTGCGGTGAGAG-3′; Ig3: 5′-GCATGTTTCCTTTCCCAATG-3′; X14: 5′-CATTCGTATTTTTTGACCCAGACC-3′. The PCR consisted of 35 cycles for the DNA analyses and 22 cycles for the cDNA.

Southern Blot Analysis

Hybridization was performed with DNA isolated from different tissues as described previously [23]. Total DNA was digested with *Hin*dIII and run on 0.8% agarose gels, transferred onto nylon filters (Hybond N, Amersham), and fixed by UV light. The ^{32}P-labeled PCR fragment containing a part of the coding region of the *set* gene (primers set 5 and 6) was used as hybridization probe.

Northern Blot Analyses

About 10 μg of total cellular RNA was separated on a 1% agarose formaldehyde gel and transferred to a Hybond-N+ membrane (Amersham) in 25 mM potassium phosphate

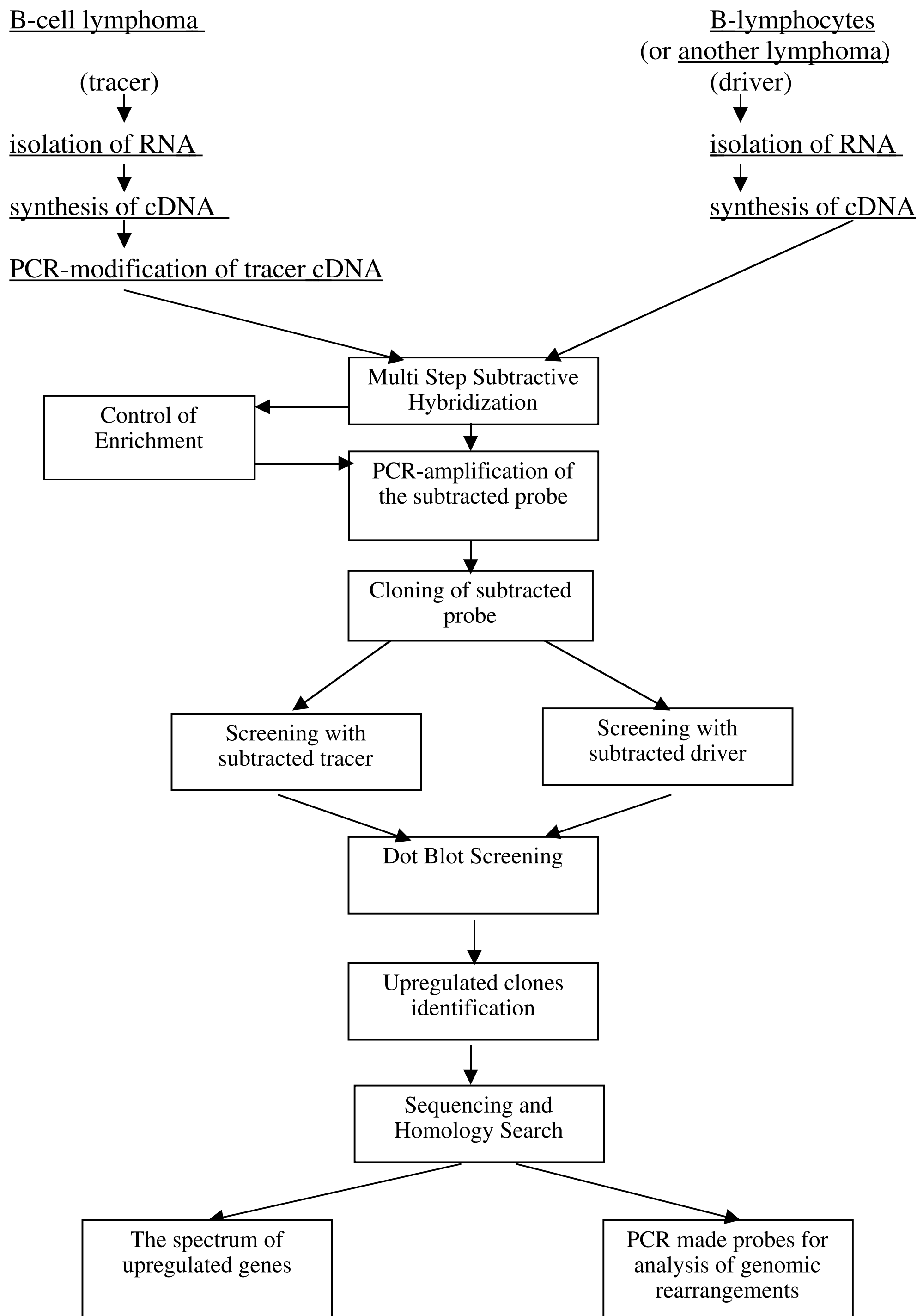

Figure 1. *Schematic representation of the approach.*

(pH 6.5). Membranes with RNA were UV-irradiated and hybridized with ^{32}P-labeled *Sal*I fragments of cDNA clones generated by subtraction coupled with differential screening. As a control, we used a ^{32}P-labeled β-actin PCR amplification product which is 658 bp in size [4].

Sequencing of DNA

Plasmid-cloned cDNA was sequenced as described [26] using Amersham Quick-Denature Plasmid-Sequencing Kit. Search for similarity of the subtracted sequences to known sequences was performed with the BLAST DataBase.

Results

Subtracted Lymphoma-Specific cDNA Libraries of AIDS-Associated B-Cell NHL

To perform the subtractive hybridization experiments, total RNA from tumor cells of two AIDS associated B-NHLs was isolated. According to their histomorphology and immunophenotype, over 90% of cells in these tumors was composed of B cells contaminated with a small percentage of infiltrating macrophages and T cells. Lymphoma A was classified as centroblastic and lymphoma B as immunoblastic (Table 1). RNA from peripheral blood B cells of a healthy donor was taken as control RNA. The cDNA synthesized from mRNA of lymphoma cells was used as tracer, cDNA synthesized from mRNA of normal B cells was used as driver.

Lymphoma-specific sequences were 40× enriched by the subtractive hybridization. After subtractive hybridization and subsequent PCR amplification of the subtracted material, the two libraries were constructed by cloning the enriched and amplified cDNA sequences into pBlueScriptSK vector.

To eliminate contaminating "nonlymphoma-specific" clones in the subtracted libraries, a two-step differential screening was introduced. The first step of the screening of the subtracted cDNA library was based on the screening of colonies of transformed bacteria by hybridization [23]. At this step of the differential screening procedure, we analysed 200 and 500 clones of the subtracted cDNA libraries from lymphomas A and B, respectively. The cDNA clones selected at the first step were then used for the second step differential screening (Figure 2). Strong hybridization signals to graded amounts of subtracted cDNA indicate a high specificity of the respective clones (Figure 2*A*). The difference in the strength of the hybridization signals between subtracted cDNA sequences and total tracer cDNA (Figure 2*A* and *B*) corresponds to the enrichment of labeled "lymphoma-specific" cDNA sequences in the subtracted cDNA. The observed differences in the strength of hybridization signals between tracer (total cDNA from the lymphoma) and driver (total cDNA from normal B cells) indicated the differential expression level of the respective genes (Figure 2*B* and *C*). Scanning of the autoradiographic images obtained after reduced exposure times (when compared to that on Figure 2) allowed to determine semiquantitatively the upregulation of genes with this selection procedure. After the second step differential screening, we identified 21 positive clones out of 200 clones from the subtracted cDNA library of lymphoma A and 16 clones out of 500 clones from the subtracted cDNA library of lymphoma B. Thus, about 4–10% of the clones of these libraries contained cDNAs sequences representing differentially expressed genes. Most of the genes selected by this approach were upregulated by a factor of 3–14.

The size of the cloned cDNA ranged from 200 to 800 bp. These cDNA inserts were partially sequenced and the sequences were analyzed with the BLAST DataBase.

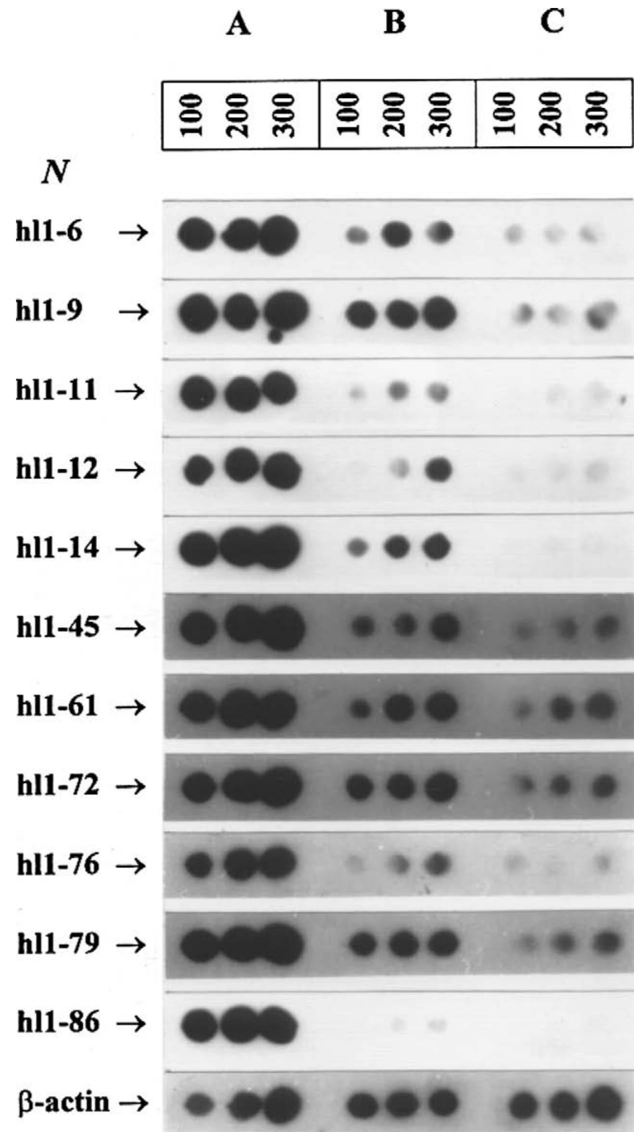

Figure 2. *Dot blot hybridization of the second step differential screening of the subtracted cDNA libraries: The ^{32}P-labeled cloned cDNA sequences were hybridized to graded amounts (in ng) of total subtracted cDNA (lane A), tracer cDNA (lane B), and driver cDNA (lane C).*

The Subtracted cDNA Library from the Centroblastic Lymphoma

Sequences lacking homology to known genes. Out of 21 sequenced lymphoma A–specific cDNA sequences, nine of them could not be easily assigned to sequences deposited in gene databases (Table 2). These cloned cDNA sequences were not unique, although some of them showed nucleotide sequence similarity to each other of up to 78%. We could not decide whether these sequence differences are caused by the error-prone PCR or are indicative of a family of closely related genes.

Clones hL1-2 and hL1-10 with lymphoma A–specific cDNAs were homologous to previously described rat and human cDNA sequences (EST), respectively.

Table 2. Homology Search with Differentially Expressed Subtracted cDNA of a Human Centroblastic Lymphoma (Lymphoma A).

Clone Number	Accession Number	Size of the Sequenced Fragment (bp)	Related Gene or cDNA	Accession Number	Similarity	
					bp	%
hL1-1	Y16696	139	Unknown	–	–	–
hL1-2	Y16715	161	EST	AIO50976	104	95
hL1-5	Y16713	255	Unknown	–	–	–
hL1-6	Y16702	194	λ2(3)IgL	X51755	71	84
hL1-8	Y16712	183	Unknown	–	–	–
hL1-9	Y16700	203	λ1IgL–set	X51755/M93651	48/154	96/97
hL1-10	Y16708	272	EST	AI377400	229	95
hL1-11	Y16697	108	Unknown	–	–	–
hL1-12	Y16714	110	*IFN-ind*	U22970	36	97
hL1-14	Y16709	152	*set*	M93651	152	97
hL1-15	Y16706	143	Unknown	–	–	–
hL1-35	Y16711	109	Unknown	–	–	–
hL1-45	Y16703	108	Unknown	–	–	–
hL1-55	Y16710	100	Unknown	–	–	–
hL1-61	Y16704	245	*ND-I*	V00662	117	95
hL1-72	Y16701	199	Unknown	–	–	–
hL1-76	Y16699	131	*ND-IV*	V00662	51	94
hL1-79	Y16707	129	*ND-IV*	V00662	128	99
hL1-86	Y16705	278	*IFN-ind*	U22970	45	97
hL1-98	Y16698	203	λ1Ig–set	X51755/M93651	48/154	96/97
hL1-101	Y17174	250	16S rRNA	V00662	247	99

cDNA with known sequences. Sequence similarities of lymphoma A–specific subtracted cDNA to already known genes or cDNAs are shown in Table 2. Homologous sequences were up to 250 bp in size and similarities ranged from 84% to 99%. Among these were clones homologous to the human *IgL* lambda 1 and 2 chain genes, to a human and monkey interferon-inducible gene (*INF-ind*), to the human mitochondrial NADH dehydrogenase subunits I and IV (*ND-I* and *ND-IV*) genes, and *16S rRNA* genes.

Three most abundantly represented clones (hL1-9, hL1-14, and hL1-98) from the subtracted cDNA library generated from the lymphoma A gave strong differential signals. They appeared to be homologous to the 3′-untranslated region of the *set* oncogene [27]. The sequences of *set*-specific

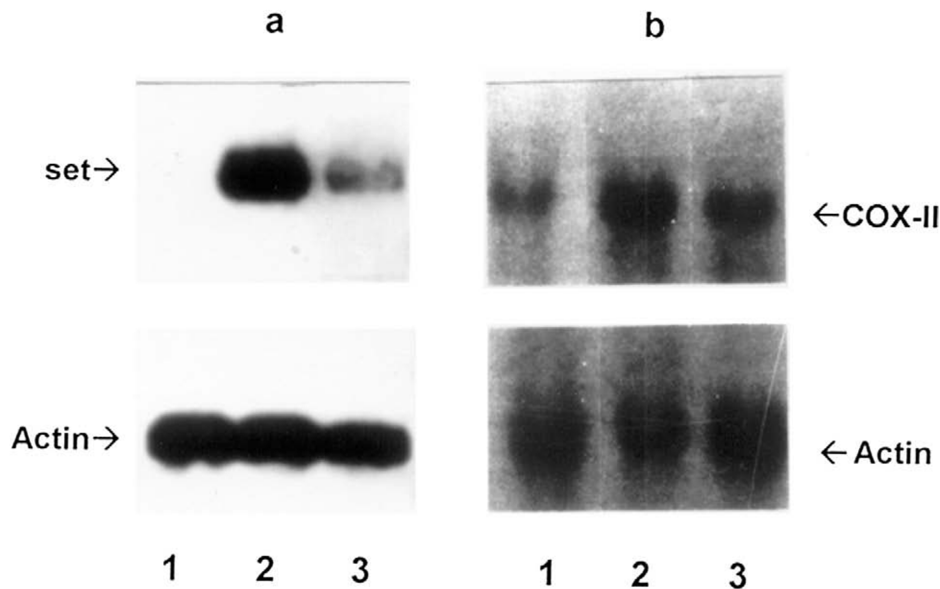

Figure 3. *Northern blot analysis to confirm differential transcription of lymphoma-specific genes. A ^{32}P-labeled pL1-14 (plasmid containing cDNA homologous to the 3′-untranslated region of the set gene) (a) and a PCR fragment of the COX-II gene (b) were hybridized to RNA isolated from naive normal B lymphocytes (lane 1), lymphoma A (lane 2), and lymph node (lane3). As a control, rehybridization with a ^{32}P-labeled PCR fragment of β-actin gene was used (on the bottom).*

Table 3. Homology Search with Differentially Expressed Subtracted cDNA of a Human Immunoblastic Lymphoma (Lymphoma B).

Clone Number	Accession Number	Size of the Sequenced Fragment (bp)	Related Gene or cDNA	Accession Number	Similarity	
					bp	%
hL2-201	Y17170	422	16S rRNA	V00662	349	99
hL2-222	Y17172	230	*ND-IV*	V00662	175	98
hL2-229	Y17171	364	AS-VI	V00662	328	99
hL2-253	Y17173	329	*ND-IV*	V00662	194	99
hL2-254	Y17174	243	16S rRNA	V00662	242	98
hL2-264	Y17176	259	*ND-IV*	V00662	246	98
hL2-274	Y17175	196	*COX-II*	V00662	180	97
hL2-378	Y17177	95	*CYTb*	V00662	78	92
hL2-383	Y17178	298	*ND-IV*	V00662	296	98
hL2-494	Y17179	264	AS VI	V00662	264	95
hL2-498	Y17180	265	*ND-IV*	V00662	208	98
hL2-500	Y17170	227	16S rRNA	V00662	225	99
hL2-501	Y17170	302	16S rRNA	V00662	299	99
hL2-502	Y17170	205	16S rRNA	V00662	203	99
hL2-503	Y17170	274	16S rRNA	V00662	271	99
hL2-504	Y17170	255	16S rRNA	V00662	252	99

cDNAs from lymphoma A comprise a sequence of the 3′-untranslated region adjacent to the open reading frame of the *set* gene. A more detailed sequence analysis revealed that such a transcript could emerge in case an internal poly(A) stretch (within the 3′-untranslated region of the *set* mRNA) anneals with *the* oligo(dT) primers that initiate the cDNA synthesis.

Multiple *set*-specific cDNA clones in the lymphoma-specific cDNA library are additional evidence for a transcriptional upregulation of this gene. To determine the relative levels of *set* sequence containing transcripts in lymphoma A and of those found in normal B lymphocytes and in lymph nodes, we performed Northern blot analyses with several RNA preparations (Figure 3*A*). The results indicated that the 3′ noncoding region of *set* gene is about 10-fold more abundantly transcribed in lymphoma cells than in the lymph nodes of a naive monkey; it was not detected in B lymphocytes of a naive animal under our experimental conditions.

The Subtracted cDNA Library from the Immunoblastic Lymphoma

Each lymphoma B–specific cDNA identified by subtractive cloning is homologous to already known genes or cDNA. Sequence similarities are shown in Table 3. The homologous sequences were up to 349 bp long and have sequence similarities of 92–99%. Each of the 16 cDNA clones analyzed so far represents sequences of mitochondrial genes. They code for 16S rRNA, cytochrome *c* oxidase II (COX-II), ATP synthase VI (AS-VI), ND-IV, and cytochrome *b* (CYTb). The clones from the subtracted lymphoma B cDNA library with sequence homologies to the *ND-IV* and *16S rRNA* genes were overrepresented (five and seven cDNA clones, respectively) and both the 16S rRNA and ND-IV cDNA were also present in the subtracted, lymphoma A–specific cDNA library (Table 2).

Abundantly Transcribed Genes Common to Both Lymphomas

Bearing in mind that subtractive cloning reveals only that limited set of genes changes significantly its transcription levels, we performed dot blot hybridization to look for transcriptional upregulation of mitochondrial genes in both lymphomas. To determine the abundant transcription of several genes in lymphomas A and B, we took as probes the cDNA clones hL2-274 (*COX-II*), hL2-229 (*AS-VI*), hL2-378 (*CYTb*) and hL1-98 (*set*). As a control, we used a PCR fragment from the mitochondrial *ND-II* gene — an apparently not overexpressed gene in both lymphomas gene (has not been detected by subtractive cloning). Each probe was hybridized to graded cDNA amounts in the same way as it was done during the second step differential screening (see Figure 2). The results of these experiments are shown in Table 4. It became evident that the mitochondrial *16S rRNA*, *COX-II*, and *ND-IV* genes are abundantly transcribed in both lymphomas and thus may be essential for malignization. In contrast, the upregulation of the *AS-VI* and *CYTb* genes seemed to be specific for lymphoma B only. There

Table 4. Upregulated Mitochondrial Gene Transcription in Two AIDS-Associated Human NHL Detected by Dot Blot Hybridization*.

Genes/Probes	Centroblastic (Lymphoma A)	Immunoblastic (Lymphoma B)
16S rRNA	+	+
Cytochrome *c* oxidase II	+	+
ATP synthase VI	–	+
Cytochrome *b*	–	+
NADH dehydrogenase I	+	+
NADH dehydrogenase IV	+	+
NADH dehydrogenase II	–	–

*As in Figure 1.
(+) Upregulation; (–) no changes.

Table 5. Homology Search with Differentially Expressed Subtracted cDNA of a Human Centroblastic Lymphoma (Lymphoma A) in Comparison with Immunoblastic Lymphoma (Lymphoma B).

Clone Number	Size of the Sequenced Fragment (bp)	Related Gene or cDNA	Accession Number	Similarity	
				bp	%
hss1-204	137	*ND-I*	V00662	84	83
hss1-206	107	Unknown	–	–	–
hss1-209	88	Unknown	–	–	–
hss1-219	144	*set*	M93651	128	96
hss1-221	98	*ND-IV*	V00662	47	100
hss1-225	105	λIgL	X51755	54	100
hss1-250	143	*set*	M93651	139	97
hss1-254	161	*ND-IV*	V00662	161	95
hss1-255	171	*set*	M93651	147	97
hss1-257	65	*set*	M93651	65	95
hss1-301	33	*IFN-ind*	U22970	26	100
hss1-316	77	*IL4R*	X52425	55	92
hss1-328	128	*TAP2*	U07844	94	95
hss1-332	256	Unknown	–	–	–

was no detectable upregulation of the *ND-II* gene in both lymphomas.

The results of the dot blot hybridizations were confirmed by Northern blot analyses with subtracted cDNA clones as probes; results for the *COX-II* gene transcription in lymphoma A are shown in Figure 3*B*.

Abundantly Transcribed Genes Different in Two Lymphomas

Apart from the genes overexpressed in both lymphomas, we have found a number of genes overexpressed only in the centroblastic lymphoma (see Tables 2 and 3). In order to demonstrate the real difference between two types of lymphomas and to estimate the adequacy and potentials of the subtractive hybridization method applied, we have carried out an additional subtraction between cDNA from the centroblastic lymphoma as tracer and cDNA from the immunoblastic lymphoma as driver. The

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hl1-9:  203   ctggttgtcaatgaggatatttattggggtttcatgagtgcggtgagag 155
hl1-98: 203   ctggttgtcaatgaggatatttattggggtttcatgagtgcggtgagag 155
λ1Ig:   4464  ctggttgtcaatgaggatatttattggggtttcatgagtgcagggagaa 4416
                     primer Ig1⇒           primer Ig2⇒

hl1-9:  154   gctggagagtgctaggcccgtgtgtagtagtgcatagaattctagctttt 105
hl1-98: 154   gctggagagtgctaggcccgtgtgtagtagtgcatagaattctagctttt 105
set:    1161  gctggagggtgctaggcccctgtgtagtagtgtatagaattctagctttt 1210
                           primer set2⇒

hl1-9:  104   ttcctcctttctctgtatattgggctcagagagtacactgtgtctctatg 55
hl1-98: 104   ttcctcctttctctgtatattgggctcagagagtacactgtgtctctatg 55
set:    1211  ttcctcctttctctgtatattgggctcagagagtacactgtgtctctatg 1260

hl1-9:  54    tgaatatggacagttagcatttaccaacatgtctctgtctactttctctt 5
hl1-98: 54    tgaatatggacagttagcatttaccaacatgtctctgtctactttctctt 5
set:    1261  tgaatatggacagttagcatttaccaacatgtatctgtctactttctctt 1310
                ⇐ primer set3              ⇐ primer set1

hl1-9:  4     gttt 1
hl1-98: 4     gttt 1
set:    1311  gttt 1314
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Figure 4. *Nucleotide sequences of lymphoma A–specific cDNA clones consisting of set and IgL gene sequences. Sequences of the cDNA clones hL1-9 and hL1-98 have similarity to the C-region of the IgL gene and to the 3′ untranslated region of the set gene (shaded area). Base pairs, which are different in cDNA clones, and the set and IgL genes are printed in bold. Primer pair set1 and set2 detects only set sequences; primer pair set1 and Ig1 detects fusion products of the IgL gene and the set gene.*

data obtained are presented in Table 5. It can be seen that the spectrum of genes overexpressed in lymphoma A as compared to lymphoma B is similar to that revealed earlier on closer examination of the overexpressed genes spectra in these lymphomas obtained by subtractive hybridization with cDNA of B lymphocytes as driver (Tables 2 and 3). The subtractions revealed that the *INF-ind* gene, the *set* gene, the *IgL* genes, and the *ND-I* and *ND-IV* genes are upregulated in the centroblastic lymphoma as compared to the immunoblastic one. In addition, two new genes [the interleukin 4 receptor (*ILR4*) gene and the ABC transporter protein 2 located in human MHC class II (TAP2) gene] and some unknown genes were revealed.

Analysis of Fused Transcripts Containing Partial Sequences of the Set Gene

Sequencing of the cloned *set*-like cDNAs (lymphoma A) revealed that apart from 154 bp of the 3′ region of the *set*

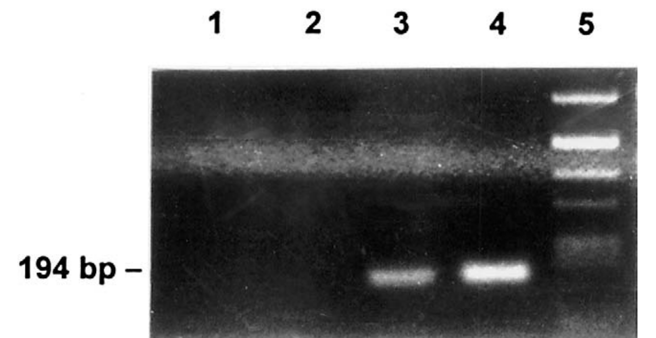

Figure 5. *PCR analyses to detect a fused set–IgL cDNA: A primer pair amplifying a set–IgL fusion was derived from the cDNA clone hL1-9 (set1 and Ig1, see Figure 3). The PCR was performed with total cDNA from normal B lymphocytes (lane 1), from lymphoma B (lane 2), from lymphoma A (lane 3), and with cDNA of clone hL1-98 as a positive control (lane 4). Lane 5, molecular weight marker (AluI digest of pBR322).*

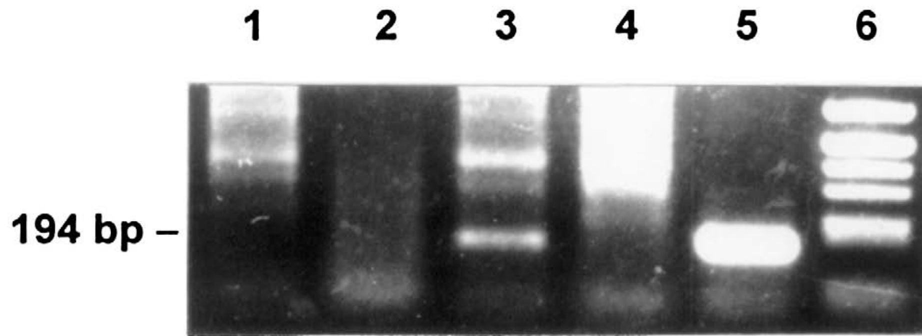

Figure 6. *PCR analyses to detect a fused IgL–set sequence in genomic DNA. To confirm a genomic rearrangement, we used the same primer pair as in the experiments shown in Figure 5. The PCR was performed with genomic DNA from normal B lymphocytes (lane 1), from lymphoma A (lane 3), from lymphoma B (lane 4), and from spleen as a negative control (lane 2). As a positive control, clone hL98 was included (lane 5). Molecular weight marker: AluI digest of pBR322 (lane 6).*

gene, some clones contained adjacent sequences not related to the *set* gene (Figure 4). In two cDNA clones hL1-9 and hL1-98, the *set*-like sequence was fused to a sequence approximately 50 bp in size having high similarity to the 3′ terminal part of the *IgL* lambda gene.

To detect cloning artefacts, we repeatedly prepared total cDNA from lymphomas A and B and from nonmalignant B cells. These preparations were then analyzed by a site-specific PCR designed to detect fusions between the *IgL*- and *set*-specific sequences. We obtained the expected 194-bp amplification product only with cDNA from lymphoma A using the set1 and Ig1 primers (Figure 5, lane 3) confirming the presence of this fused mRNA. With the set1 and X14 primers, which should detect the fused sequence present in clone hL1-14, no amplification product was found neither in lymphomas A and B cDNAs nor in normal B cell cDNA (data not shown). Therefore, it is likely that the cDNA of clone hL14 is a cloning artefact. Thus, only lymphoma A contained an mRNA species combining the 3′-untranslated regions of the *IgL* and *set* genes.

A genomic rearrangement in the centroblastic lymphoma is responsible for the fusion of the *IgL* and *set* genes. Clones hL1-9 and hL1-98 contained fused sequences. This finding prompted us to obtain evidence for a genomic rearrangement between parts of the *set* gene and the *IgL* gene in lymphoma cells. To do this, we performed a site-directed PCR analysis with genomic DNA with the set1 and Ig1 primers located within the *set* gene and the *IgL* gene, respectively. The amplification product, 194 bp in size, was synthesized only with genomic DNA of lymphoma A (Figure 6, lane 3). The PCR performed with genomic DNA of lymphoma B, nonmalignant B cells, and spleen tissue did not result in the expected 194-bp amplification product. The detection of such a highly specific amplification product synthesized from genomic DNA of lymphoma A is clear evidence for a chromosomal rearrangement in this lymphoma.

Having the sequences of the C-region of the *Ig lambda1* gene and the *set* mRNA in mind (the complete *set* gene sequence is unknown), we constructed several pairs of primers for PCR amplification of sequences spanning the region of the genomic rearrangement in DNA of lymphoma A (Figure 7). The primers were derived from the putative fused DNA sequence, i.e., in each primer pair, one primer was specific for the C-region of the *Ig lambda1* gene whereas the other one was specific for the *set* mRNA region. After amplification and subsequent gel electrophoresis coupled

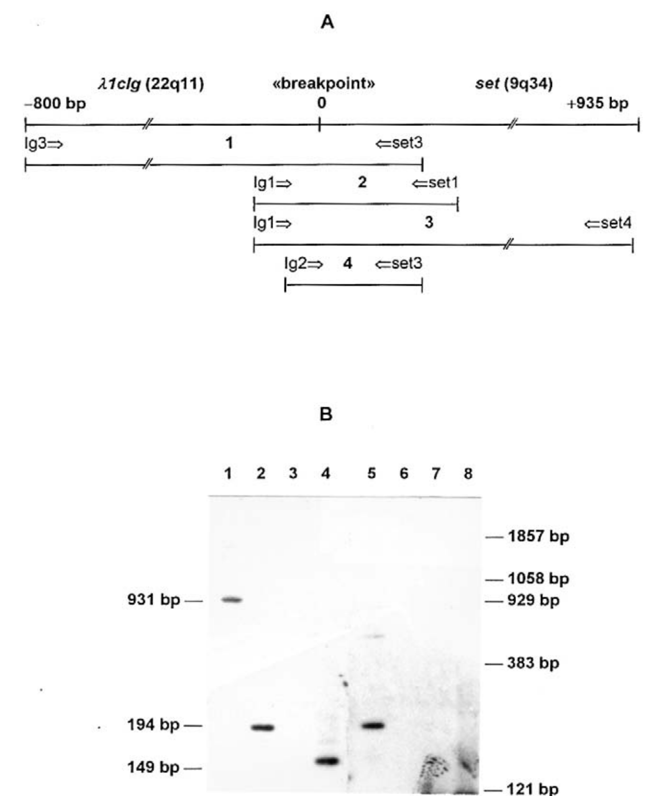

Figure 7. *(A) The putative genomic fusion sequence. The IgL–set sequence, the localization of the break point, and the diagnostic PCR primers. The expected PCR products and corresponding primer pairs are indicated: 1, Ig3–set3; 2, Ig1–set1; 3, Ig1–set4; 4, Ig2–set3. (B) PCR analyses and Southern blot hybridization. PCR was performed on genomic DNA of lymphoma A (lanes 1–4) and normal B lymphocytes (lanes 6–8). The hybridization was performed with the ^{32}P-labeled PCR fragment (primers Ig1 and set1) amplified from the hL1-98 clone. Lanes 1 and 6, Ig3–set3 primer pair; lanes 2 and 7, Ig1–set1 primer pair; lane 3, Ig1–set4 primer pair; lanes 4 and 8, Ig2–set3 primer pair. Lane 5 contains a positive control (PCR fragment Ig1–set1). Molecular weight marker: AluI digest of pBR322.*

with a Southern hybridization using labeled 194-bp PCR fragment (a PCR with the Ig1–set1 primer pair), several predictable bands were observed (931-bp PCR fragment with the Ig3–set3 primer pair, lane 1; 194-bp PCR fragment with the Ig1–set1 primer pair, lane 2; 149-bp PCR fragment with the Ig2–set3 primer pair, lane 4). These bands are characteristic for the observed fused chromosomal DNA region and led to a more precise and detailed information about the genome translocation in lymphoma. In case of B lymphocytes, expected bands were absent.

To further characterize this genome rearrangement, a Southern blot analysis of genomic DNA of both lymphomas and B lymphocytes was performed (Figure 8). The 587-bp PCR fragment corresponding to a coding region of the *set* mRNA (the set4–set5 primer pair and DNA from B lymphocytes) was used as a labeled probe. The Southern blot analysis revealed three major hybridization signals corresponding to common genome fragments of all three DNA preparations. There is also a low molecular weight fragment seen only with DNA of lymphoma B and B lymphocytes, but not with DNA of lymphoma A. To interpret the obtained hybridization patterns, we made a computer search for *set*-like DNA sequences in the BLAST DataBase. With sequences in the database, we searched for *Hind*III fragments correlating to the restriction pattern shown in Figure 8. Indeed, we found several genomic sequences whose *Hind*III fragments were similar in size to the fragments found; i.e., a 8059-bp fragment of chromosome 17 (acc. no. 005666); a 8059-bp DNA fragment of X chromosome (acc. no. Z95126); a 5426-bp DNA fragment of 21 chromosome (acc. no. 004106); and a DNA fragment with an approximate size of 4400 bp of chromosome 3. Although we performed the computer search with updated databases, we did not find any homology to the *set*-like fragment of approximately 2000 bp, which had not been observed in DNA of lymphoma A. We concluded that this fragment is related to a *set* sequence, which is not yet available. The absence of the 2000-bp fragment in lymphoma A may also be indicative of a genomic rearrangement leading to the loss of a *Hind*III restriction site(s).

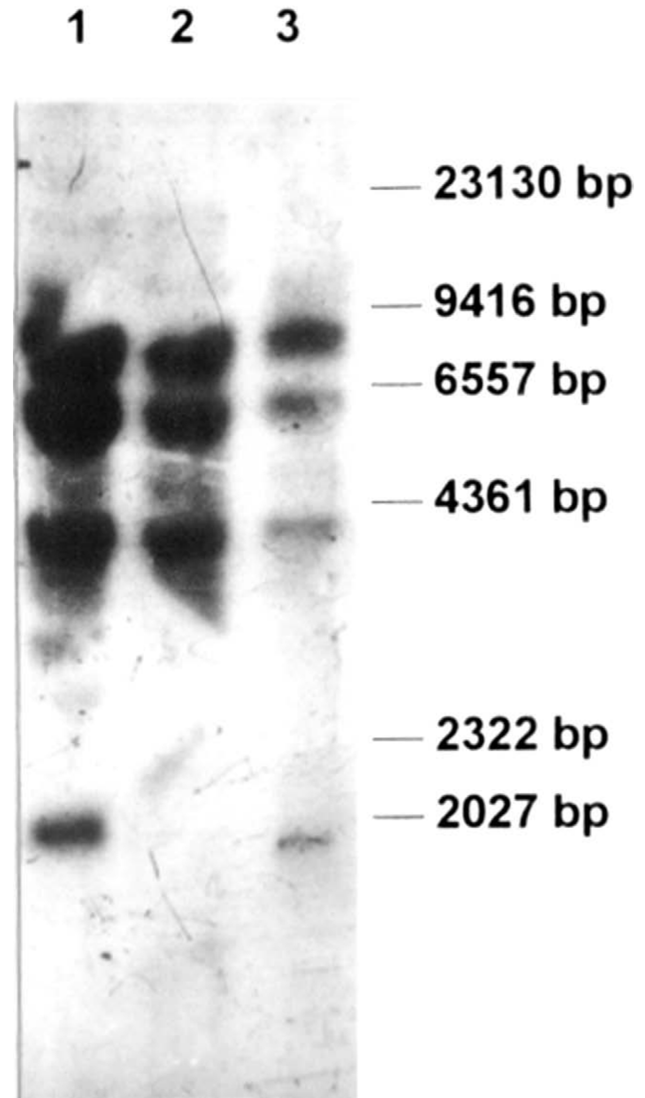

Figure 8. *Southern blot hybridization of HindIII restricted genomic DNA. DNA isolated from naive B lymphocytes (lane 1), lymphoma A (lane 2), and lymphoma B (lane 3) were hybridized with a ^{32}P-labeled PCR fragment produced by amplification with the set4–set5 primers located inside of the coding sequence of the set gene. Marker, lambda DNA/HindIII (lane 4).*

Discussion

Our studies aimed at the detection of specifically expressed or upregulated genes in AIDS-associated B-NHL. We used a full-scale subtractive cloning procedure to obtain a broadened spectrum of genes preferably expressed in lymphomas.

In the centroblastic lymphoma, we detected cDNA clones containing sequences homologous to several known nuclear and mitochondrial genes. Among the lymphoma-specific cDNA clones, eight proved to have no sequence homology to any known sequence in the GenBank database. They may represent novel genes whose transcription was upregulated only in human lymphoma tested. Interestingly, some of these clones share up to 78% sequence identity. However, we cannot yet decide whether these genes are variants of one single gene or are closely related unique genes. Furthermore, we identified two lymphoma-specific cDNA clones (hL1-2 and hL1-10) containing substantial sequence homology with ESTs.

Among the upregulated genes in the AIDS-associated lymphoma A, we detected the *INF-ind* gene (clones hL1-12 and 86). An enhanced expression of this *INF-ind* gene had been found to be specifically transcribed in liver tissue of a hepatitis C virus–infected chimpanzee [28,29] and in B-NHL of SIV-infected monkeys [21]. According to recent data, *IFN-ind* genes have been previously implicated in the regulation of cell growth and malignant transformation [30]. A recently discovered INF-induced factor represses the expression from the HIV-1 long terminal repeat [31].

In both the centroblastic and the immunoblastic AIDS-associated lymphomas, we detected several upre-

gulated mitochondrial genes. The overexpression of the mitochondrial *COX-II*, *ND-IV*, and *16S rRNA* genes in both lymphomas is remarkable. The detection of cDNA having homology to the *16S rRNA* may be due to a fused transcript encoding parts of *ND-I* and *16S rRNA* (these genes are located nearly in mitochondrial genome). Such transcripts have been already detected in cancer cells [9].

Analyses of genes differentially expressed in different types of malignant cells have already revealed altered transcription levels of several mitochondrial genes, including the *ND-IV* and the *COX-II* genes [9–11]. Similarly, we also have detected such an abundant transcription of these mitochondrial genes in SIV-associated monkey lymphomas [21]. It is important that *ND-II* gene used as a control was not overexpressed. It indicates that the observed enhanced expression of some mitochondrial genes' subunits was not due to the increased number of mitochondria in metabolically active cells.

Our experimental findings concerning SIV- and HIV-associated lymphomas, as well as current reports, let us suppose that the lymphomagenesis as well as cancerogenesis in general may be linked to a modulated synthesis of some enzymes belonging to the oxidative phosphorylation pathways or their subunits.

As to EBV- and HIV-specific sequences, we were not able to select any corresponding cDNA by our subtractive technique although EBNA2 and p24 proteins, respectively, were detectable by immunohistochemistry (Table 1). Perhaps, the abundance of these viral mRNA species in lymphoma cells is extremely low and the number of clones analyzed so far is not sufficient to reveal them.

Special experiments revealed that 1) the differences between the spectra of the genes overexpressed in the two lymphomas found using earlier versions of subtraction are reliable; and 2) the method applied here provides a comprehensive spectrum of upregulated genes in the lymphomas.

A high percentage of cDNA clones in the subtracted library of lymphoma A contains an *IgL–set* gene fusion sequence. According to differential screening and analyses of cDNA, the *set* gene appeared to be only slightly upregulated in lymphoma B. Quite similar to the *set* gene expression in HIV-associated lymphomas, we detected an upregulation of the *set* gene in diffuse large B cell monkey lymphomas associated with SIV [21]. There is no conclusive evidence for any function of the *set* gene in these lymphomas. Its gene product is a 39-kDa nuclear phosphoprotein playing a role in DNA replication and inhibition of protein phosphatase 2A [32,33].

One of the most important findings concerning lymphoma A is that *set* gene-like sequence appears to be rearranged. The chromosomal breakpoints are supposed to be located 3′ to the Cλ1 region of the *IgL* gene and in the 3′-untranslated region of the *set* gene. This primary genetic event may be analogous to the *IgL* gene sequences translocated into the c-*myc* gene [7,34]. Previously, part of the *set* gene was found to be fused to the *can* oncogene in acute undifferentiated leukemia cells by a chromosomal translocation [27]. Our study demonstrated for the first time the existence of elevated levels of fused transcripts containing a 3′-untranslated element of the *set*-like gene and the *IgL* gene in HIV associated B-NHL.

Chromosomal translocations involving different loci are already recognized as common specific cytogenetic abnormalities in lymphoma cells [3]. Studying such translocations allowed to identify several genes involved in lymphomagenesis, e.g., *bcl-6* [35], *ALK* and *NPM* [5], cycline D [36]. It is known that the gene locus of the lambda-chain of Ig is prone to genetic rearrangements in follicular lymphomas [7,32]. Since the *set* gene is located on chromosome 9 (q34) and the *IgL* gene on chromosome 22 (q11), the sequence identified may result from a t(9;22)(q34;q11) translocation event. Unfortunately, about 10 sequences with similarity to the 3′-untranslated region of the *set* gene are supposed to be present in the human genome [27]. Thus, further studies are needed to confirm the exact site and chromosome where the rearrangement took place. Nevertheless, our subtractive hybridization technique allowed us to detect a genomic rearrangement never observed previously with less sophisticated methods. Recently, molecular studies of the Philadelphia-like translocation t(9;22)(q34;q11) in a follicular lymphoma indicated an IgL-mediated rearrangement of an unknown gene at 9q34 that may be involved in the lymphomagenesis [37]. According to our data, the unknown gene sequence might belong to one of the multiple *set* gene copies.

The genomic rearrangement of the *set* and the *IgL* genes may be a primary genetic event causing the high level of fused transcripts in the centroblastic lymphoma A. In immunoblastic lymphoma B, there was no evidence for an *IgL–set* translocation, although *set* gene–containing transcripts were present.

Conclusion

We succeeded in identifying a panel of genes not yet reported to be differentially expressed in AIDS-associated or in other human NHLs. Moreover, we have detected a unique genomic rearrangement between *set* gene-like sequences and the *IgL* gene in a centroblastic AIDS-associated NHL. However, further studies are necessary to determine whether these genes contribute to lymphoma development and can be used for lymphoma classification or as therapeutic targets.

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