Biochemical and Biophysical Research Communications **283,** 12–18 (2001)
doi:10.1006/bbrc.2001.4731, available online at http://www.idealibrary.com on IDEAL®

Genetic Imbalances in Preleukemic Thymuses

Myriam Verlaet,*,†,[1] Valérie Deregowski,‡ Ghislaine Denis,*,† Chantal Humblet,†,§ Marie-Thérèse Stalmans,*,† Vincent Bours,‡ Vincent Castronovo,†,¶ Jacques Boniver,*,† and Marie-Paule Defresne†,§

**Laboratory of Pathological Anatomy and Cytology, ‡Laboratory of Medical Chemistry and Medical Oncology, ¶Metastasis Research Laboratory; §Laboratory of Histology and Cytology; †Centre de Recherche en Cancérologie Expérimentale, University of Liège, CHU, B-4000 Liège, Belgium*

Received March 5, 2001

To understand the molecular mechanisms involved in preleukemia, the suppression subtractive hybridization method was used in a murine radiation-induced thymic lymphoma model. Seventeen mRNAs overexpressed in preleukemic thymuses were identified: mouse laminin binding protein (p40/37LBP), E25 protein, *Rattus norvegicus* clone BB.1.4.1, profilin, poly(A) binding protein (PABP), mouse high mobility group protein 1, topoisomerase I, clusterin, proteasome RC1 subunit, rat prostatein C3 and C1 subunits; two ESTs and four unknown genes. The overexpression of PABP, clusterin, profilin, and the p40/37LBP mRNAs was confirmed in preleukemic thymuses and can be related to some cellular events observed during the preleukemic period, i.e., alterations of cell cycle and apoptosis properties. The p40/37LBP and 67-kDa laminin receptor proteins were upregulated during the preleukemic period. The data suggest that additional studies on p40/37LBP and 67-kDa laminin receptor regulation are required to evaluate their potential role in the lymphoma prevention by TNF-α and IFN-γ.

Key Words: **suppression subtractive hybridization (SSH); preneoplasm; leukemia; p40/37LRP; laminin receptor.**

Murine radiation induced thymic lymphoma is a model of multistep carcinogenesis first developed by Kaplan (1). This model has been used to analyze, at the cellular level, the progression leading to the development of tumors (2–11). Tumors are preceded by a 3–9 months preleukemic period during which preleukemic cells (PLCs) appear (2, 7, 8). These cells require the thymic microenvironment for their neoplastic transformation: indeed, on the contrary to leukemic cells, capable to induce tumors when injected both in thymus bearing and in thymectomized recipients, the preleukemic cells only induce tumors when grafted into thymus-bearing syngeneic recipients, but never in thymectomized mice (4, 7–9). As a result of the leukemogenic treatment, thymic lymphopoiesis is profoundly altered and the properties of the thymic epithelium are disturbed (3). In some thymocytes the suicide program is activated but these cells seem to be rescued from apoptosis by environmental thymic factors (5). Furthermore, the cell cycle properties are abnormal (6).

The gene(s) responsible for these alterations are not yet identified. They are limited data regarding on the identification and quantitation of gene expression in pretumoral lesions (11–13). In this study, we used a PCR-based cDNA subtraction method called suppression subtractive hybridization (SSH) (14) to identify cDNAs of differentially expressed genes. Target cDNAs present in the preleukemic cells but absent (or present at lower levels) in control cells were amplified. This procedure led to the identification of 17 genes overexpressed in thymuses of preleukemic mice 30 days after the last irradiation, including 4 unknown genes and 2 expressed sequence tags, that have never previously been associated with thymic transformation. Eleven genes matches to known mRNAs. One of these latter, the p40/37LBP, was selected to confirm differential gene and protein expression during the preleukemic period. The 37-kDa laminin binding protein (37LBP) is a building element of the non-integrin 67-kDa laminin receptor (67LR) and exhibits multiple functions in various aspects of cell growth, embryonic development and cancer progression (15, 16). Previous reports have demonstrated the up-regulated expression of the 37LBP

Abbreviations used: SSH, suppression subtractive hybridization; 37LBP, 37-kDa mouse laminin binding protein; 67LR, 67-kDa laminin receptor; PABP, poly(A) binding protein; EST, expressed sequence tags.

[1] To whom correspondence should be addressed at CNCM, University of Liège, Place Delcour 17, B-4000 Liège, Belgium. Fax: 00 32 4 366 59 53. E-mail: M. Verlaet@ulg.ac.be.

0006-291X/01 $35.00

and 67LR in various human cancer cells and tissues (17–21), such as colon, breast, ovary, lung, pancreas, gastric, endometria, hepatocellular carcinoma prostate, thyroid, and melanoma. Other studies have also demonstrated that 37LBP functions as a ribosomal protein (22). We reported a differential p40/37LBP and 67LR proteins expression during the preleukemic period.

MATERIALS AND METHODS

Mice. One-month-old female congenic Thy-1.1 C57BL/Ka mice (called BL/1.1 for convenience) were initially obtained from M. Lieberman (Stanford University, CA). They were raised in the animal house of the Liège University. Animal care was provided in accordance with procedures outlined in the "Law for Care and Use of Laboratory Animals" (Arrété Royal, November 14, 1993, Belgium).

Irradiations. For lymphoma induction, mice received whole-body irradiation of 1.75 Gy once each week for 4 weeks. The irradiation was delivered by an X-ray apparatus (Stabilivolt Siemens, 190 kB, 18 mA, HVL of 0.5 mm Cu, and focal distance of 35 cm) at a dose rate of 1.6 Gy/minute.

Oligonucleotides. Sequences described by Diatchenko (14) were used (Eurogentec, Liège, Belgium).

RNAs extraction. Total RNAs were purified from thymuses of control mice and preleukemic mice 30 days after the last irradiation using homogenization with guanidine isothiocyanate and phenol/chloroform extraction (23). Poly(A)$^+$ RNA was purified from total RNA by PolyATtract mRNA Isolation System IV (Promega, Leiden, The Netherlands).

cDNAs preparation. The ds cDNAs were synthesized from 2 μg of poly(A)$^+$ RNAs of the thymuses using the Marathon cDNA Amplification Kit (CLONTECH, Leusden, The Netherlands) and 1 ng of cDNA synthesis primer. First- and second-strand cDNA synthesis and blunt-ending of DNA ends by T4 DNA polymerase were carried out according to the manufacturer's protocol. The resulting cDNAs were digested by *Rsa*I (Roche Diagnostics, Mannheim, Germany) for 3 h, phenol extracted and ethanol precipitated. The final concentration of driver was ≈300 ng/μl.

Adapters ligation. One hundred nanograms of digested preleukemic cDNAs were ligated with adapter 1 and adapter 2 in separate 10 μl ligation reactions containing 2 μM adapter 1 and 2 μM adapter 2 at 16°C overnight, using 0.5 units of T4 DNA ligase (Life Technologies, Merelbeke, Belgium) in the buffer supplied from the manufacturer. After ligation, 1 μl of 0.2 M EDTA was added and the samples were heated at 70°C for 5 min to inactivate the ligase and stored at −20°C.

Subtractive hybridization. The subtractive hybridization was carried out according to Diatchenko's protocol (14).

PCR amplification. The primary PCR was conducted in 50 μl: 1 μl of diluted subtracted cDNAs, 1 μl of PCR primer P1 (5 μM), 1 μl of PCR primer P2 (5 μM), and 47 μl of PCR master mix (Roche Diagnostics, Mannheim, Germany). PCR was performed with the following parameters: 75°C for 7 min; 30 cycles at (91°C for 30 s; 68°C for 30 s; 72°C for 2.5 min); and a final extension at 68°C for 7 min. The amplified products were diluted 10-fold in deionized water. One microliter was used in a secondary PCR, in which PCR primers 1 and 2 were replaced with nested primers PN1 and PN2.

Cloning and sequencing of subtracted cDNAs. Products from the secondary PCR were inserted into pCRII using a TOPO TA cloning kit (Invitrogen, Groningen, The Netherlands). Plasmid DNAs were prepared using QIAprep Miniprep (QIAGEN, Leusden, The Netherlands) according to the manufacturer's protocol. DNA sequencing was performed with T7 Sequencing Kit (Amersham Pharmacia Biotech, Essex, UK).

Nucleic acid homology searches were performed using the BLASTN and FastA programs through GCG Belgian Embnet.

Northern blot analysis. Twenty micrograms of total RNA was subjected to electrophoresis and blotted. *Eco*RI linearized plasmids (50 ng) for mvx2006, mvx2008, mv12x1, mvx2015, mvx11052, mvx11075 and mvx11115 clones were eluted from agarose gels and purified by Quantum Prep Gel Slice kit (Bio-Rad, Nazareth, Belgium). The labeling reaction was performed with Ready-To-Go DNA labeling kit (-dCTP) (Amersham Pharmacia Biotech, Essex, UK) according to the manufacturer's protocol. Linearized plasmid for 37-LBP served as substrate for riboprobe synthesis in a reaction containing 200 ng od DNA, 2.5 mM ATP, GTP, CTP, 100 μM UTP, 50 μCi [α-^{32}P]UTP, 100 mM dithiothreitol, 1 U RNasin ribonuclease inhibitor (Promega); 5 U T7 RNA polymerase and 1× transcription buffer (Roche Diagnostics). The probes were purified on Quick Spin Column (Amersham Pharmacia Biotech, Essex, UK). Radioactive bands intensities were measured by densitometry by use Bio-Profil (Vilber Lourmat, Marne La Vallée, France). Densitometric values were normalized to the respective 28S intensity for each samples ± SEM.

RT-PCR. The cDNAs for RT-PCR were prepared using Superscript II RNase H$^-$ Reverse Transcriptase (Life Technologies, Merelbeke, Belgium) and 250–500 ng of total RNA from preleukemic and control thymuses 30 days after the last irradiation, using standard conditions recommended by the manufacturer (Life Technologies, Merelbeke, Belgium). The PCR was carried out using PCR Master (Roche Diagnostics, Mannheim, Germany) with a standard aliquot of the cDNA preparations and 20 pg of each primer pair: HPRT sense 5′-GTTGGATACAGGCCAGACTTTGTTG-3′; 5′-antisense GATTCAACTTGCGCTCATCTTAGGC-3′ (9); cytochrome *c* oxidase subunit 1 sense 5′-TCCACTGATTCCCACTATTC-3′; antisense 5′-GAGCCTATAGAGGAGACTGT-3′; E25 sense 5′-AATCCTTCCGCCTTAGACGC-3′; antisense 5′-CCCCTTTCCCTTTCCTTTTCC-3′; mvx11115 clone sense 5′-TGATGGCCCTCTGGGAAGAGT-3′; antisense 5′-TCTCCAGCAGGGAGTCGATGCG-3′; mv12x1 clone sense 5′-TACAACAGCCACCACAGTGTCC-3′; antisense 5′-GCTGCCTCTTGCCAAAGTTC-3′; mvx11075 clone sense 5′-GTGCTTTGCCCATCATTG-3′; antisense 5′-GCTTCTTCTTCTGCTCCACTTC-3′. Cycling conditions were: 95°C 5 min, 95°C 1 min/50°C 1 min/72°C 1 min (35 cycles), and 72°C 5 min. A negative control without cDNA was included for each PCR reaction. The results are expressed as semiquantitative values based on the ratio of the intensity of studied genes and HPRT PCR products analyzed on ethidium bromide-stained agarose gels using the BIO-PROFIL gel analyzer system (Vilbert Lourmat, Marne La Vallée, France).

Western blot analysis of 37-kDa laminin receptor precursor and 67-kDa laminin receptor. Thymic proteins were prepared as described by Tasken *et al.* (24). Protein concentrations were determined using the Bio-Rad protein Assay according to Bradford method. Twenty micrograms of total protein were boiled in 25 μl of sample buffer (Tris–HCl 50 mM pH 6.75, glycerol 10%, SDS 2%, β-mercaptoethanol 5%, bromophenol blue 0.05%) for 5 min. Twenty microliters were subjected to electrophoresis on 10% SDS–PAGE. Proteins were transferred to Immobilon-P membranes (Millipore, Belgium) and probed with polyclonal rabbit anti-37LBP and anti-67LR antibody (from Metastasis Research Laboratory). The signal was then visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech, Essex, UK). To normalize protein loadings, the membranes were reprobed with an affinity-isolated antigen-specific anti-actin antibody (Sigma-Aldrich, Bornem, Belgium). Band intensities were measured by densitometry by use of the BIO-PROFIL gel analyzer system (Vilbert Lourmat, Marne La Vallée, France). Densitometry values for 37LBP and 67LR were normalized to the respective actin intensity for each sample ± SEM.

TABLE 1

Identity of Differentially Expressed Clones

Clone	Accession number[a]	Size[b] (bp)	Clone identity	mRNA size (bp)[c]	Accession number	Total no. of individual cDNA clones[d]	Method used to verify differential expression[e]
Mvx2002	AJ279846	96	HMGI	1225	X80457	2	—
Mvx2005	AJ279847	212	PABP	2244	X65553	2	NB
Mvx2006	AJ279848	195	profilin	756	X14425	1	NB
Mvx2010	AJ279849	192	EST	466	AW125381	2	—
Mvx2011	AJ279850	212	Topoisomerase 1	3512	D10061	1	—
Mvx2015	AJ279851	70	E25 mRNa	1635	L38971	1	NB, RT-PCR
Mvx2017	AJ279852	56	C3 subunit	509	J00772	5	—
Mv12x1	AJ279833	295	EST	557	AW123170	1	NB, RT-PCR
Mv10x100	AJ279951	108	Proteasome subunit RC1	1047	D10729	1	—
Mv12x100	AJ279952	83	Unknown	—	—	1	—
Mv100x17	AJ279834	240	SGP-2	1857	M16975	2	RT-PCR
Mvs11002	AJ279835	170	37LBP/p40	1031	X06406	1	NB
Mvx11007	AJ279836	333	SGP-2	1857	M16975	2	RT-PCR
Mvx11052	AJ279837	95	Clone BB1.4.1.	1876	U40628	12	NB, RT-PCR
Mvx11072	AJ279838	93	Clone BB1.4.1.	1876	U40628	4	RT-PCR
Mvx11075	AJ279839	255	Unknown	500	M33330	1	NB, RT-PCR
Mvx11076	AJ279840	125	Clone BB1.4.1.	1876	U40628	3	RT-PCR
Mvx11081	AJ279841	224	C1 subunit	412	V01545	1	—
Mvx11089	AJ279842	129	Unknown	—	—	1	—
Mvx11090	AJ279843	210	Unknown	—	—	1	—
Mvx11098	AJ279844	254	SGP-2	1857	M16975	2	RT-PCR
Mvx11115	AJ279845	259	SGP-2	1857	M16975	1	NB, RT-PCR

[a] Nucleotide sequences reported in this study have been submitted to the EMBL database and assigned these accession numbers.
[b] Size of cDNAs cloned in the differential screening without the size of the primers on each side.
[c] Size of the identified mRNAs.
[d] Number of sequenced clones derived from the same gene.
[e] Method used to confirm differential gene expression: Northern blotting (NB) or RT-PCR.

RESULTS

Identification of Differentially Expressed mRNAs

The cDNA clones representing transcripts preferentially expressed in preleukemic thymuses but absent or present at lower levels in control thymuses, were recovered by suppression subtractive hybridization (SSH) as described under Materials and Methods. Tester cDNAs were prepared from thymic mRNAs of mice sacrificed 30 days after a leukemogenic irradiation. Driver cDNAs, prepared from thymuses of control mice, were used in excess during cDNA subtraction. The nucleotide sequence of 48 chosen subtracted cDNA clones was determined and 17 individual sequences were identified. The differentially expressed genes were grouped into three different classes based on computer searches against the GenBank and EMBL databases: (1) novel sequences; (2) previously identified genes with relatively uncharacterized function; (3) known genes with previously characterized function. The results are summarized in Table 1.

Among the four novel identified genes, one (mvx11075) shared partial homology at the nucleotide level with sequence of mouse insulinoma mRNA *rig*, which is robosomal protein S15 (25) and the three other clones represented unknown genes in the DNA databases.

Among the two existing sequences in the EST databases with uncharacterized function and overexpressed in preleukemic thymuses, the first one (mvx2010) matched a cDNA sequence described as being expressed in mouse brain, mammary gland, irradiated colon cells, lymph nodes and fetus (26); and the second one mv12x1 is expressed in mouse skin and embryo (26).

Among the known genes identified in our screening, four genes identified have functions that, until now, have no relation with the neoplastic transformation. The first one (mvx2015) is the mouse E25 mRNA, described as being associated with chondro-osteogenic differentiation (27). The second one (mvx11052 or mvx11072) is the *Rattus norvegicus* clone BB.1.4.1. unknown Glu-Pro dipeptide repeat protein mRNA (28): it has a high nucleotide homology with the cytochrome *c* oxidase subunit 1 which is a mitochondrial protein (29). With this respect, it is interesting to notice that a mitochondrial gene is upregulated in the pretumoral period and that the activity of the cytochrome *c* oxidase is consistent with the cytochrome *c* oxidase subunit 1 overexpression in the preleukemic thymuses (Verlaet

et al., in preparation). The third and fourth genes are the poly(A) binding protein (PABP) (mvx2005) and the profilin (mvx2006), which will be discussed later on.

The other genes identified have been previously reported to be associated with several aspects of tumorogenicity in general. The High mobility group (HMG) nonhistone chromosomal protein I, a member of the HMG-I(Y) family of architectural transcription factors, has been linked with human cancer and with neoplastic and metastasic phenotypes (30–32). The Topoisomerase I, involved in DNA replication, is known to be associated with solid tumors (33–35). Elevated levels of the rat prostatic binding protein subunits C3 and C1, androgen-responsive *in vivo* rat prostate, are observed in prostate cancer (36). Clusterin expression confined to surviving cells following induction of apoptosis (37). Rat proteasome subunit RC1 involved in the ubiquitin pathway, which is often the target of cancer-related deregulation (38), showed a high expression mRNA in the Yoshida sarcoma (39). The cytoplasmic ribosomal-associated protein p40, that has been also cloned as the metastasis-associated 67-kDa membrane-associated laminin receptor precursor (37LRP) (40), is frequently upregulated in several cancers in association with the metastatic phenotype of the lesion. Surprising, no thymic genes were detected. Probably, it is the result of the nonexhaustive selection of positive clones.

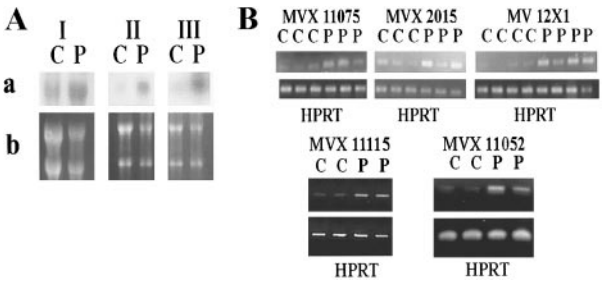

FIG. 1. Confirmation of genes expression. (A) Northern blot analysis of thymus RNAs after a leukemogenic irradiation. Thymuses were removed at the day of sacrifice (30) and were processed for Northern blotting (see Materials and Methods). (a) I, mvx11002 (37LBP/p40); II, mvx2006 (profilin); III, mvx2005 (PABP) levels in age-matched control (C) and preleukemic (P) thymuses RNAs 30 days after the last irradiation for 2 experiments. (b) Representation of ethidium bromide staining of transferred 28S and 18S ribosomal RNAs. (B) RT-PCR analysis. Evaluated expression of mvx11115 (Clusterin), mvx11052 (Clone BB1.4.1.), mvx11075 (unknown), mvx2015 (E25), mv12x1 (EST) and HPRT mRNAs using reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR products, visualized on ethidium bromide-stained gels, in preleukemic (P) thymuses RNAs 30 days after the last irradiation and in age-matched control (C) thymuses for 2, 3, or 4 RNA sets experiments.

Confirmation of Gene Expression by Northern Blot and RT-PCR Analyses

Northern blot was used to assess the relative abundance of the mvx11002 (37LBP/p40), mvx2006 (profilin) and mvx2005 (PABP) transcripts with respect to 28S ribosomal transcripts in two sets of preleukemic and control thymuses RNAs (Fig. 1A). The Northern blot for the mvx11002 clone was performed with the 37 LRP probe (see Materials and Methods). The mvx2006 and mvx2005 cDNA fragments were used as probes for Northern analysis. We found that the relative levels of mvx11002 (37LRP/p40), mvx2006 (profilin) and mvx2005 (PABP) with respect to 28S were higher in preleukemic thymuses that in controls. The ^{32}P-labeled riboprobe signals were respectively 2.1, 1.6 and 7.2 times higher in preleukemic thymus total RNAs as compared to control thymus total RNAs. Similar approaches were followed for mvx11115 (clusterin), mvx11052 (cytochrome *c* oxidase subunit 1), mvx11075 (unknown), mvx2015 (E25) and mvx12x1 (EST) clones. However, no signal was observed in control and preleukemic thymus total RNAs by Northern blot. Then the RT-PCR technique was used to evaluate the expression of mvx11115 (clusterin), mvx11052 (cytochrome *c* oxidase subunit 1), mvx11075 (unknown), mvx2015 (E25) and mv12x1 (EST) clones in preleukemic and control thymuses. A representative experiment with RT-PCR products from two 30-day-old preleukemic thymuses and two control thymuses was analyzed (Fig. 1B). The amounts of different transcripts were higher in the preleukemic thymuses as compared with the control thymuses. In this model, no lymphoma was induced in 5 to 10% of treated mice (41). This observation could explain the lower expression for MVX2015 in the second preleukemic sample.

Expression of 37LBP/p40 and 67LR Proteins during the Preleukemic Period

37LBP/p40 (mvx11002) is a cytoplasmic-ribosomal protein associated with the translation machinery and its increased expression plays an important role in the invasive and metastatic phenotype as a putative basic building block of a receptor to accommodate the extracellular matrix protein laminin, the 67LR (laminin receptor). The 67LR is overexpressed in several solid tumors including breast, colorectal, gastric and cervical carcinoma in correlation with the invasive and metastatic phenotype (42). We therefore looked for their expression in the preleukemic thymuses. Western blot analyses were performed with preleukemic and control thymus protein pools. As shown in Figs. 2A and 2B, 37LBP/p40 expression was higher in preleukemic thymuses compared to controls: respectively, 1.5, 3.0, 2.25, and 2.0 times higher at day 30, 45, 60, and 90 after the last irradiation. However, when a thymic lymphoma developed, the 37LBP/p40 polypeptide expression was 4 times higher than in control thymuses (43). Western blot analysis (Figs. 2A and 2C) using an anti-67LR antibody demonstrated that an increased 67LR expression was observed in parallel with the age in both

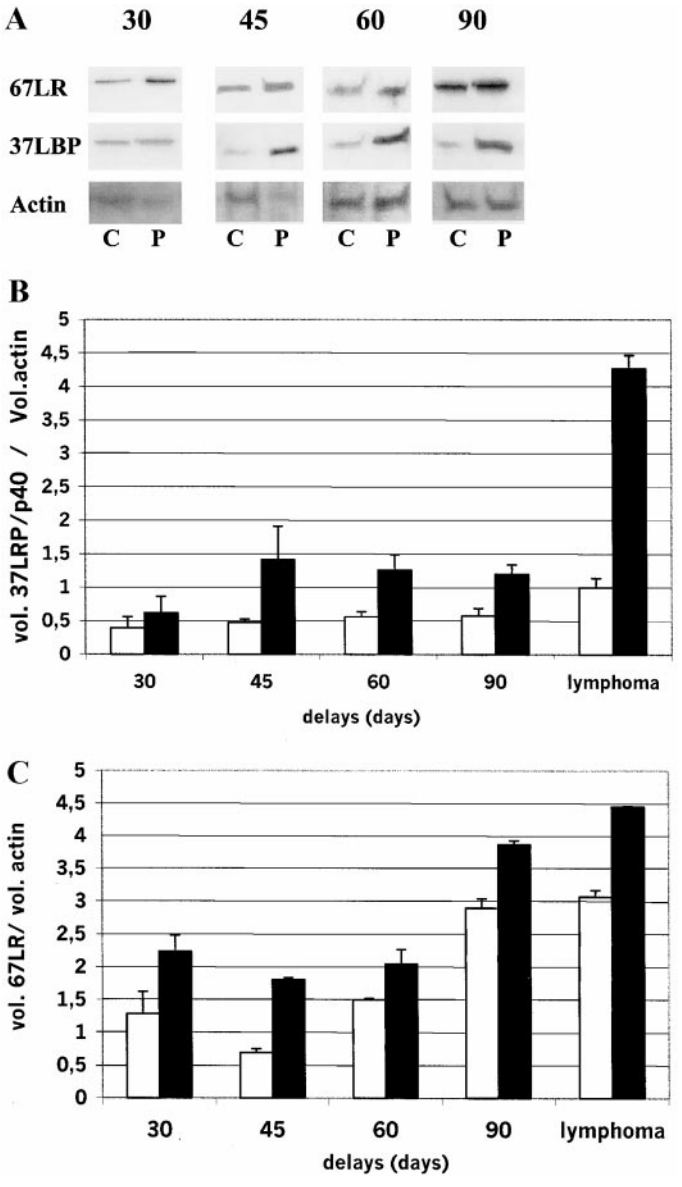

FIG. 2. (A) Western blots analysis of mice after a leukemogenic irradiation. Thymuses were removed at the day of sacrifice (30, 45, 60 and 90 days after the last irradiation) and were processed for Western blotting (see Materials and Methods). 37LRP/p40, 67LR and actin levels were estimated in preleukemic (P) thymuses extracts 30, 45, 60, 90 days after the last irradiation and in age-matched control (c) thymuses extracts. (B) Densitometry analysis. The panel plots the ratio 37LRP/p40 on actin volumes analysis at each delay postirradiation in control (open bars) and preleukemic (filled bars) thymuses. (C) 67LR protein expression densitometry analysis. The panel plots the ratio 67LR on actin volumes analysis at each delay post-irradiation in control (open bars) and preleukemic (filled bars) thymuses.

experimental conditions (control and irradiation). However, this receptor was overexpressed in all postirradiation time intervals examined as compared to control conditions. As showed in Fig. 2C, the differential expression was respectively 1.7 and 2.6 times higher 30 and 45 days after the last irradiation, i.e., the preleukemic period. Subsequently, on days 60 and 90, the overexpression was respectively only 1.4 and 1.3 times higher. In lymphomas, 67LR overexpression was weaker than 37LBP overexpression.

DISCUSSION

The identification of genes specifically overexpressed or repressed in pretumoral tissues is a key step towards the understanding of the malignant transformation process. In this study a PCR-based subtractive hybridization method was applied and 17 genes whose expression levels are altered were identified in a well-characterized model of radiation-induced carcinogenesis (1). This model is particularly interesting given the multistep progression observed during the carcinogenetic process (4).

Moreover, only a few studies have so far used tissues as starting material, most studies comparing gene expression between cell lines. Analysis of genes in tissues, offers two major advantages. Firstly, growth factors and other additives used in culture media do not alter gene expression patterns. Secondly, cell–cell and cell–matrix interactions can be taken into account. Indeed, in our model, it is known that preleukemic cells are dependent upon host factors and thymic microenvironment for their further evolution into thymic lymphoma (3, 7–9).

The differential screening approach allowed us to identify cDNAs whose expression is upregulated in preleukemic thymuses 30 days after the last irradiation, a time when the probability to observe many characteristic events of the preleukemic period is very high (2, 3, 6). The low differences of expression observed in this study are not surprising, many others studies demonstrated low assessed expression differences with this method (12, 27, 44, 45). The overexpression of PABP, clusterin, profilin and p40/37LBP, confirmed by Northern blot or RT-PCR, can be related to some cellular events observed during the preleukemic period.

(A) The poly(A) binding protein (PABP) is involved in the transport of poly(A)$^+$ RNA into the cytoplasm, the degree of mRNA polyadenylation, the mRNA half-life and the modulation of translation initiation and/or reinitiation events by interacting with ribosomal subunits (46). Its overexpression in our model may be merely reflective of higher proliferation of some preleukemic cells.

(B) The clusterin, also called SGP-2 or TRPM2, is a secreted sulfated glycoprotein whose expression is confined to surviving cells following the induction of apoptosis *in vitro,* suggesting that it is involved in cell survival rather than death (37): it could reflect or contribute to the abnormal cell survival observed in preleukemic thymuses.

(C) The profilin is a cytoskeleton protein regulated by PIP2 and forms a 1:1 complex with G-actin (47). It may represent a link between matrix–cell receptor–cytoskeleton and cell signal transduction to the nucleus, and then play a role in cell proliferation and apoptosis (48, 49).

(D) The cytoplasmic ribosomal-associated protein p40/37LBP is frequently upregulated in several cancers in association with the metastatic phenotype of the lesion (40).

The data demonstrated that p40/37LBP and the receptor form of 67 kDa are overexpressed during the preleukemic period with a peak 45 days after the last irradiation. Interestingly, the overexpression of 67LR mRNA in preleukemic thymuses 30 days after the last irradiation was also observed by using arrayed filters (Atlas Array from Clontech) (data not shown). The *in situ* hybridization and immunohistochemical studies (data not shown) demonstrated a localization of the p40/37LBP in thymocytes but also in stromal cells. Both types of cells expressed p40/37LBP and 67LR as confirmed by a Western blot on epithelial cell lines, a primary stromal cell culture and thymocytes (data not shown). The distribution pattern of 37LBP/p40 polypeptide as observed by immunohistochemistry revealed the presence of the protein in the cortex but also in the medulla of thymuses in areas where 37 LBP transcripts were observed. The same distribution was observed by immunohistochemical studies for the laminin 1 protein (data not shown).

This overexpression of p40/37LBP during preleukemic period could explain some cellular events observed during preleukemia. First of all, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), which have been shown to inhibit the development of radiation-induced thymic lymphomas (50–52) can induce a down-regulation of the p40/37LBP gene promoter activity, hence a decrease of the p40/37LBP mRNA levels, which is accompanied by a reduced expression of the two forms of the protein, the p40/37LBP and the 67 LR (53). The protective effect of TNF-α and IFN-γ could be, at least in part, the result of the p40/37LBP and 67LR decrease. Next, our previous results (3, 5) led us to suggest that a subset of thymocytes, destined to die in preleukemic thymuses, is rescued from their final fate by yet unknown microenvironmental factors. Kaneda *et al.* (54) observed, using antisense or sense p40 cDNA that the loss of p40 expression in HeLa cells can inhibit cell growth and induce apoptotic cell death. Thus a p40/37LBP and/or a clusterin increase (see above) could prevent apoptosis of a subset of cells that are destined to die in the preleukemic thymuses.

In addition, the p40/37LBP localized on the 40S ribosome in mouse sarcoma and erythroleukemia cells (55) is involved in the translation process (22). Two others genes overexpressed in preleukemic cells 30 days after the last irradiation are also involved in translation processes: *rig*, which is the rat ribosomal protein S15 (25) and PABP, which modulates the translation initiation and/or reinitiation events by interacting with ribosomal subunits (46). The increased expression of p40/37LBP, *rig* and PABP in tumor cells agrees with the observation that ribosomal protein synthesis increases in growing cells as compared with resting cells (25). As p40/37LBP is a basic building block of the 67LR, it might provide a link between protein synthesis and cell-surface events.

In conclusion, we have demonstrated that PCR-based subtractive hybridization method in combination with the conventional Northern blot and RT-PCR is highly efficient to detect differentially expressed genes. Some transcripts identified in this study (p40/37LBP, profilin, clusterin, poly(A)-binding protein) represent valuable candidates genes for further functional analysis in prelymphomas and should be informative in studying the biology of the tumour: indeed they seem to participate in a network between cytoskeleton, cell membrane, protein synthesis and signal transduction, and could thus be involved in mediating proliferation and/or apoptosis inhibition. The significance of the overexpression of other transcripts has still to be elucidated.

ACKNOWLEDGMENTS

We are particularly grateful to Dr. S. L. Giannini, T. Grisar, and B. Lakaye for the helpful suggestions and to M. C. Petit for her technical assistance. This work was supported by a grant from the Centre Anticancéreux près l'Université de Liège. M. Verlaet, V. Deregowski, and G. Denis were supported by a predoctoral fellowship from the National Funds for Scientific Research (FNRS). V.B. is a Senior Research associate of FNRS.

REFERENCES

1. Kaplan, H. S. (1967) *Cancer Res.* **27,** 1325–1340.
2. Boniver, J., Decleve, A., Lieberman, M., Honsik, C., Travis, M., and Kaplan, H. S. (1981) *Cancer Res.* **41,** 390–392.
3. Defresne, M. P., Greimers, R., Lenaerts, P., and Boniver, J. (1986) *J. Natl. Cancer Inst.* **77,** 1079–1085.
4. Haran-Ghera, N. (1978) *Leuk. Res.* **2,** 11–21.
5. Humblet, C., Deman, J., Franzen, R., Greimers, R., Boniver, J., and Defresne, M. P. (1995) *Stem Cells* **13**(Suppl. 1), 129–135.
6. Rongy, A. M., Humblet, C., Lelievre, P., Greimers, R., Defresne, M. P., and Boniver, J. (1990) *Thymus* **16,** 7–27.
7. Muto, M., Kubo, E., and Sado, T. (1985) *J. Immunol.* **134,** 2026–2031.
8. Muto, M., Sado, T., Hayata, I., Nagasawa, F., Kamisaku, H., and Kubo, E. (1983) *Cancer Res.* **43,** 3822–3827.
9. Muto, M., Kubo, E., and Sado, T. (1987) *Cancer Res.* **47,** 3469–3472.
10. Muto, M., Kubo, E., Kamisaku, H., and Sado, T. (1990) *J. Immunol.* **144,** 849–853.
11. Muto, M., Chen, Y., Kubo, E., and Mita, K. (1996) *Jpn. J. Cancer Res.* **87,** 247–257.

12. Tchernitsa, O. I., Zuber, J., Sers, C., Brinckmann, R., Britsch, S. K., Adams, V., and Schafer, R. (1999) *Oncogene* **18,** 5448–5454.
13. Coggin, J. H., Jr., Rohrer, J. W., and Barsoum, A. L. (1997) *Int. J. Radiat. Biol.* **71,** 81–94.
14. Diatchenko, L., Lau, Y. F., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E. D., and Siebert, P. D. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6025–6030.
15. Menard, S., Tagliabue, E., and Colnaghi, M. I. (1998) *Breast Cancer Res. Treat.* **52,** 137–145.
16. Satoh, K., Narumi, K., Abe, T., Sakai, T., Kikuchi, T., Tanaka, M., Shimo-Oka, T., Uchida, M., Tezuka, F., Isemura, M., and Nukiwa, T. (1999) *Br. J. Cancer* **80,** 1115–1122.
17. Castronovo, V., Campo, E., van den Brule, F. A., Claysmith, A. P., Cioce, V., Liu, F. T., Fernandez, P. L., and Sobel, M. E. (1992) *J. Natl. Cancer Inst.* **84,** 1161–1169.
18. Hipfel, R., Schittek, B., Bodingbauer, Y., and Garbe, C. (2000) *Br. J. Cancer* **82,** 1149–1157.
19. Montuori, N., Muller, F., De Riu, S., Fenzi, G., Sobel, M. E., Rossi, G., and Vitale, M. (1999) *J. Clin. Endocrinol. Metab.* **84,** 2086–2092.
20. Pelosi, G., Pasini, F., Bresaola, E., Bogina, G., Pederzoli, P., Biolo, S., Menard, S., and Zamboni, G. (1997) *J. Pathol.* **183,** 62–69.
21. Waltregny, D., de Leval, L., Menard, S., de Leval, J., and Castronovo, V. (1997) *J. Natl. Cancer Inst.* **89,** 1224–1227.
22. Demianova, M., Formosa, T. G., and Ellis, S. R. (1996) *J. Biol. Chem.* **271,** 11383–11391.
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *in* Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
24. Tasken, K., Skalhegg, B. S., Solberg, R., Andersson, K. B., Taylor, S. S., Lea, T., Blomhoff, H. K., Jahnsen, T., and Hansson, V. (1993) *J. Biol. Chem.* **268,** 21276–21283.
25. Kitagawa, M., Takasawa, S., Kikuchi, N., Itoh, T., Teraoka, H., Yamamoto, H., and Okamoto, H. (1991) *FEBS Lett.* **283,** 210–214.
26. Bonaldo, M. F., Lennon, G., and Soares, M. B. (1996) *Genome Res.* **6,** 791–806.
27. Deleersnijder, W., Hong, G., Cortvrindt, R., Poirier, C., Tylzanowski, P., Pittois, K., Van Marck, E., and Merregaert, J. (1996) *J. Biol. Chem.* **271,** 19475–19482.
28. Geertman, R., McMahon, A., and Sabban, E. L. (1996) *Biochim. Biophys. Acta* **1306,** 147–152.
29. Ku, C. Y., Lu, Q., Ussuf, K. K., Weinstock, G. M., and Sanborn, B. M. (1991) *Mol. Endocrinol.* **5,** 1669–1676.
30. Abe, N., Watanabe, T., Sugiyama, M., Uchimura, H., Chiappetta, G., Fusco, A., and Atomi, Y. (1999) *Cancer Res.* **59,** 1169–1174.
31. Cmarik, J. L., Li, Y., Ogram, S. A., Min, H., Reeves, R., and Colburn, N. H. (1998) *Oncogene* **16,** 3387–3396.
32. Kim, S. J., Ryu, J. W., and Choi, D. S. (2000) *Korean J. Intern. Med.* **15,** 71–75.
33. Abang, A. M. (1998) *Semin. Hematol.* **35,** 13–21.
34. Arbuck, S. G., and Takimoto, C. H. (1998) *Semin. Hematol.* **35,** 3–12.
35. Hochster, H., Liebes, L., Speyer, J., Sorich, J., Taubes, B., Oratz, R., Wernz, J., Chachoua, A., Blum, R. H., and Zeleniuch-Jacquotte, A. (1997) *Clin. Cancer Res.* **3,** 1245–1252.
36. Labrie, C., Simard, J., Zhao, H. F., Pelletier, G., and Labrie, F. (1990) *Mol. Cell Endocrinol.* **68,** 169–179.
37. Viard, I., Wehrli, P., Jornot, L., Bullani, R., Vechietti, J. L., Schifferli, J. A., Tschopp, J., and French, L. E. (1999) *J. Invest. Dermatol.* **112,** 290–296.
38. Spataro, V., Norbury, C., and Harris, A. L. (1998) *Br. J. Cancer* **77,** 448–455.
39. Tsuchiya, H., Tsuchiya, Y., Kobayashi, T., Kikuchi, Y., and Hino, O. (1994) *Jpn. J. Cancer Res.* **85,** 1099–1104.
40. Rao, C. N., Castronovo, V., Schmitt, M. C., Wewer, U. M., Claysmith, A. P., Liotta, L. A., and Sobel, M. E. (1989) *Biochemistry* **28,** 7476–7486.
41. Scott, W. N., McFerran, N. V., Harriott, P., Walker, B., and Nelson, J. (2000) *Biochim. Biophys. Acta* **1481,** 25–36.
42. Gasparini, G., Barbareschi, M., Boracchi, P., Bevilacqua, P., Verderio, P., Dalla, P. P., and Menard, S. (1995) *Int. J. Cancer* **60,** 604–610.
43. Sobel, M. E. (1993) *Semin. Cancer Biol.* **4,** 311–317.
44. Oh, J. J., Grosshans, D. R., Wong, S. G., and Slamon, D. J. (1993) *Nucleic Acids. Res.* **27,** 4008–4017.
45. Kuang, W. W., Thompson, D. A., Hoch, R. V., and Weigel, R. J. (1998) *Nucleic Acids Res.* **26,** 1116–1123.
46. Muller, D., Rehbein, M., Baumeister, H., and Richter, D. (1992) *Nucleic Acids Res.* **20,** 1471–1475.
47. Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., Nakao, K., Jockusch, B. M., and Narumiya, S. (1997) *EMBO J.* **16,** 3044–3056.
48. Oren, A., Herschkovitz, A., Ben Dror, I., Holdengreber, V., Ben Shaul, Y., Seger, R., and Vardimon, L. (1999) *Mol. Cell Biol.* **19,** 1742–1750.
49. Wiedemann, P., Giehl, K., Almo, S. C., Fedorov, A. A., Girvin, M., Steinberger, P., Rudiger, M., Ortner, M., Sippl, M., Dolecek, C., Kraft, D., Jockusch, B., and Valenta, R. (1996) *J. Biol. Chem.* **271,** 29915–29921.
50. Boniver, J., Humblet, C., and Defresne, M. P. (1989) *Leukemia* **3,** 611–613.
51. Defresne, M. P., Humblet, C., Rongy, A. M., and Boniver, J. (1989) *Thymus* **13,** 201–204.
52. Humblet, C., Greimers, R., Delvenne, P., Deman, J., Boniver, J., and Defresne, M. P. (1996) *J. Natl. Cancer Inst.* **88,** 824–831.
53. Clausse, N., van den, B. F., Delvenne, P., Jacobs, N., Franzen-Detrooz, E., Jackers, P., and Castronovo, V. (1998) *Biochem. Biophys. Res. Commun.* **251,** 564–569.
54. Kaneda, Y., Kinoshita, K., Sato, M., Saeki, Y., Yamada, R., Wataya-Kaneda, M., and Tanaka, K. (1998) *Cell Death. Differ.* **5,** 20–28.
55. Auth, D., and Brawerman, G. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4368–4372.