Molecular Cloning and Characterization of the Mouse tag7 Gene
Encoding a Novel Cytokine*

(Received for publication, January 5, 1998, and in revised form, April 28, 1998)

Sergei L. Kiselev‡§¶, Olga S. Kustikova‡§¶, Elena V. Korobko‡¶, Egor B. Prokhortchouk‡,
Andrei A. Kabishev‡, Evgenii M. Lukanimidin§¶, and Georgii P. Georgiev‡‡

From the §Institute of Gene Biology, 34/5 Vavilova St., Moscow 117334, Russia and the ‡Danish Cancer Society,
Strandboulevarden 49, 7.1, DK-2100 Copenhagen, Denmark

Cloning of the mouse tag7 gene encoding a novel cytokine is described. The Tag7 protein consists of 182
amino acids. Genomic organization of the tag7 gene and its promoter region remind those of the genes of the
tumor necrosis factor locus, although the tag7 gene is not linked to this locus. The gene is located on chromo-
some 7 at the area that corresponds to band 7A3, which has genetic linkage with lupus-like disease in mouse
models. tag7 transcription is essential for lymphoid organs. It is also detected in certain areas of lungs, brain,
and intestine and in some tumors. Tag7 protein is detectable in both cell-associated and soluble forms. The
soluble form of Tag7 triggers apoptosis in mouse L929 cells in vitro and does not involve NF-κB activation.
The relationship between Tag7 and tumor necrosis factor family of ligands is discussed.

The term “cytokine” has come to be used for a diverse group of growth factors, inflammatory mediators, and hematopoietic
regulators that are distinct from more classical hormones secreted by the glands of the endocrine system. Cytokines are
proteins that act as a soluble cell to cell messengers, distinct from more classical hormones secreted by the glands of the
endocrine system. Cytokines are proteins that act as a soluble cell to cell messengers, distinguished by their high activity,
and can act in both autocrine and paracrine manners.

Neoplastic cells themselves characteristically produce cytokines spontaneously, and even in the same lineage, they can have
different cytokine expression and/or secretion profiles (1–4). Cytokines secreted by tumors could be used either as auto-
crine factors, to recruit and suppress reactive leukocytes, or to
modulate the activity of endothelial and stromal cells. The ability to release cytokine in an autocrine manner could be a
key factor in the promotion of neoplastic transformation and in permitting tumor growth in vivo (5). In their turn, cytokines
produced by the host in response to a tumor modify the scenario
created by tumor growth (6). The analysis of expression of
cytokine genes by tumors indicated that cells constitutively
produced both autostimulatory and inhibitory cytokines. Ex-
pression of several cytokines, including interleukin-1β (IL-1β),
IL-6, IL-8, IL-10, tumor necrosis factor-α, lymphotoxin-α, and
granulocyte-macrophage colony-stimulating factor by some tu-
mors has been described. Tumor necrosis factor (TNF) and
lymphotoxin-α (LT-α, also known as TNF-β) are related cyto-
kines involved in many regulatory activities (7, 8), but their
roles in the immune system although suggesting very critical
functions (9) are still enigmas. TNF and LT-α are released by a
number of tumor cells originating in mouse fibrosarcoma, ep-
ithelial human cell lines, and T-cell leukemia (10–12).

In this study, we used a pair of related mouse transplanted
 tumors with the opposite metastatic properties to identify
genes overexpressed in one of them. As a result, the tag7 gene
was cloned. It was expressed in tumor with high metastatic
potential, although no correlation with metastatic potential
was detected after study of many different tumors. The tag7
gene is preferentially transcribed in normal lymphoid and
hematopoietic cells. Tag7 is a secreted protein possessing a signif-
icient cytotoxicity realized through apoptosis.

MATERIALS AND METHODS

Cells and Cell Cultures—VMR-0, VMR-L, CSML-0, and CSML-100
cell lines were established from their respective mammary adenocarci-
noma transplanted tumors described in Ref. 13. Cells lines saved their
ability to produce tumors in syngeneic mice with low (VMMF-0 and
SBL) or with high (VMR-L and CSML-100) metastatic potential when
injected subcutaneously. Mouse spleenocytes, thymocytes, monocytes,
and peritoneal macrophages were isolated as described previously (14).
Cells were cultured in RPMI 1640 medium containing 5% FBS 100
units/ml penicillin, 100 units/ml streptomycin, 10 μM HEPES. Cells
were activated with LPS (5 μg/ml) in serum-free medium for different
time. VMR-0 cells were transfected with the pMSGneo tag7 construct or
mock-transfected using Lipofectin reagent (Life Technologies, Inc.) ac-
cording to the manufacturer’s recommendations, and clones were se-
lected on G418 resistance and maintained.

Cloning of the tag7 Gene—A fragment of the tag7 cDNA was isolated by
differential display technique essentially as described by Liang and
Pardee (15). T7-AC oligonucleotide was used as the anchoring primer,
and AATCGGGCTG was used as the arbitrary primer. A fragment of
390 bp was used as a probe for Northern blot hybridization and cDNA
library screening (16). cDNA library was prepared on polyA+ RNA
isolated from VMR-L tumors using ZAP-cDNA Gigapack Cloning kit
(Stratagene) according to the manufacturer’s recommendation. Positive
clones were purified, and the inserts were excised as pBluescript clones
using helper phage as described by the manufacturer. Few clones were
sequenced using Sequenase Version 2.0 sequencing kit (Amersham
Pharmacia Biotech) and synthetic oligonucleotide primers (Applied Bio-
systems 391 DNA synthesizer). Genomic library was constructed in the
λ FIX II vector (Stratagene) and screened according to a standard
procedure (16). The inserts were subcloned in pGEM7Z vector and
partially sequenced as described above.

RNA Hybridization—Total RNA from tumors and cell lines was

† This work was supported by International Association for the Promo-

tion of Cooperation with Scientists from the New Independent States of the
 Former Soviet Union (INTAS) Grant N1010-CT93-0029, Pharmaceut-

cal European Community Organization Grant ERB3530PL941128, and the

Danish Cancer Society, Strandboulevarden 49, 7.1, DK-2100 Copenhagen, Denmark

‡ These authors contributed equally to this work.

§ To whom correspondence should be addressed. Tel.: 7-095-1359970;
Fax: 7-095-1354105; E-mail: slk@mx.ibg.rssi.ru.

¶ This nucleotide sequence(s) reported in this paper has been submitted
to the GenBank™/EBI Data Bank with accession number(s) X86374
and X86375 (cDNA) and Y12089 (genomic DNA).

1 The abbreviations used are: IL, interleukin; TNF, tumor necrosis
factor; rTNF, recombinant human TNF; LT, lymphotoxin; LPS, lipopo-
lysaccharide; kb, kilobase pair; bp, base pair.
isolated by guanidine thiocyanate procedure, resolved on a 1.2% agarose-formaldehyde gel, and blotted onto Hybond-N as recommended by the manufacturer (Amersham Pharmacia Biotech). The EcoRI/Xhol fragment from the longest cDNA clone was labeled by random priming and used as a probe. To equalize an amount of RNA loaded on each lane, hybridizations were performed with glyceraldehyde-3-phosphate dehydrogenase DNA probe was performed. In situ hybridization was performed as described previously (17).

DNA—Oligonucleotides were synthesized corresponding to the 5’ and 3’ ends of the coding regions of the mouse tag7 gene, with BamHI and HindIII restriction sites appended to the ends of oligonucleotides. The coding region of the gene was amplified by standard polymerase chain reaction techniques, cut with BamHI and HindIII, and inserted in frame in the BamHI and HindIII sites of the pQE30 expression vector (Qiagen). For eucaryotic expression, full-sized tag7 cDNA was subcloned in NheI-Xhol sites of pBR-CMV (Stratagene) and EcoRI-BamHI sites of pM5Gneo vectors.

Chromosomal Mapping of the tag7 Gene—Fluorescence in situ hybridization on metaphase mouse chromosomes was performed by Genome Systems, Inc.

Immunological Methods—Escherichia coli recombinant Tag7 protein was expressed in M15[pREP4] (Qiagen) and purified on Ni-NTA agarose (Qiagen) as recommended by the manufacturer. Rabbit antibodies raised against recombinant Tag7 were affinity-purified on a Sepharose (Amersham Pharmacia Biotech) column with the immobilized recombinant Tag7, as recommended by the manufacturer. SDS-polyacrylamide gel electrophoresis, immunoprecipitation, and immunoblotting were performed according to standard procedures (16). The approximate amount of secreted Tag7 by VMRSX8 clone was determined by immunoblotting.

Analysis of Tag7 Multimeric Structure—Mouse splenocytes (1.5 × 10^8 cells) were treated with LPS for 18 h on two 100-mm dishes as described above. After that, Complete Mini EDTA-free protease inhibitor mix (Boehringer Mannheim) and EDTA to final concentration 1 mM were added to conditioned medium. Conditioned medium was concentrated and fractionated on Superdex 75 HR10/30 column (Amersham Pharmacia Biotech) to obtain 20 mM Tris-HCl, pH 7.5, 100 mM NaCl according to the manufacturer’s recommendation. For column calibration, bovine serum albumin (66 kDa), ovalbumin (45 kDa) (Sigma) were used. Proteins from collected fractions were precipitated with trichloroacetic acid, resolved by 15% SDS-polyacrylamide gel electrophoresis, and transferred to membrane. The membrane was then probed with anti-Tag7 polyclonal antiserum.

Tag7 Cytotoxicity Assay and Neutralization of Cytotoxic Activity—A source of the native form of the Tag7 protein, the medium conditioned by the VMR-0 pM5Gneo (VMRSX8)-transfected cells was used. L929 cells were cultured in 96-well plates at a density of 3 × 10^4 cells/well. After overnight incubation, cells were treated with actinomycin D (1 µg/ml) for 2 h at 37 °C in serum-free medium. After that, 100 µl/well of the VMRSX8-conditioned medium or VMR-0-conditioned medium were added. As indicated, recombinant human TNF (rhTNF) (Sigma) was added at a concentration of 10 ng/ml in a volume of 100 µl/well. To determine cell death, the cytotoxicity assay was used or cells were stained with trypan blue and the coded samples were counted under the microscope in a blind fashion, with a minimum of 100 cells scored for each group.

Neutralization of the cytotoxic effect of the Tag7 protein or rhTNF was performed using affinity-purified polyclonal rabbit anti-Tag7 and polyclonal anti-hTNF (Sigma) antibodies. Polyclonal antibodies were added to the VMRSX8-conditioned medium at a final concentration of 2 µg/ml, and the cytotoxic effect was determined as described above; rabbit IgG in the same concentration was used as a control.

DNA Fragmentation Analysis—DNA fragmentation analysis was performed as described (18), with modifications. In brief, 2 × 10^6 L929 cells were preincubated with actinomycin D (1 µg/ml) for 2 h at 37 °C in serum-free medium and subsequently incubated with the VMRSX8-conditioned medium or rhTNF for 5 h. Cells were harvested and lysed in 20 mM Tris-HCl, pH 8.0, 0.8% Triton X-100, 10 mM EDTA, pH 8.0. After centrifugation, DNA from the supernatant was precipitated at -20 °C by isopropanol in the presence of NaCl. DNA was resuspended in TE buffer (10 mM Tris-Cl, pH 7.4, 1 mM EDTA, pH 8.0) and treated with RNase A, and fragments were resolved in 1.8% agarose gel in TBE buffer (90 mM Tris-borate, 90 mM boric acid, 2 mM EDTA). NF-κB Activation Assay—Nuclear extracts were obtained as described in Ref. 19, and EMSA was performed according to Refs. 16 and 20. NF-κB consensus oligonucleotide (Promega) was used for gel shift assay.

Actinomycin D (1 µg/ml) for 2 h at 37 °C in serum-free medium. After that, 100 µl/well of the VMRSX8-conditioned medium or VMR-0-conditioned medium were added. As indicated, recombinant human TNF (rhTNF) (Sigma) was added at a concentration of 10 ng/ml in a volume of 100 µl/well. To determine cell death, the cytotoxicity assay was used or cells were stained with trypan blue and the coded samples were counted under the microscope in a blind fashion, with a minimum of 100 cells scored for each group.

Neutralization of the cytotoxic effect of the Tag7 protein or rhTNF was performed using affinity-purified polyclonal rabbit anti-Tag7 and polyclonal anti-hTNF (Sigma) antibodies. Polyclonal antibodies were added to the VMRSX8-conditioned medium at a final concentration of 2 µg/ml, and the cytotoxic effect was determined as described above; rabbit IgG in the same concentration was used as a control.

DNA Fragmentation Analysis—DNA fragmentation analysis was performed as described (18), with modifications. In brief, 2 × 10^6 L929 cells were preincubated with actinomycin D (1 µg/ml) for 2 h at 37 °C in serum-free medium and subsequently incubated with the VMRSX8-conditioned medium or rhTNF for 5 h. Cells were harvested and lysed in 20 mM Tris-HCl, pH 8.0, 0.8% Triton X-100, 10 mM EDTA, pH 8.0. After centrifugation, DNA from the supernatant was centrifugated at -20 °C for 10 min in the presence of NaCl. DNA was resuspended in TE buffer (10 mM Tris-Cl, pH 7.4, 1 mM EDTA, pH 8.0) and treated with RNase A, and fragments were resolved in 1.8% agarose gel in TBE buffer (90 mM Tris-borate, 90 mM boric acid, 2 mM EDTA). NF-κB Activation Assay—Nuclear extracts were obtained as described in Ref. 19, and EMSA was performed according to Refs. 16 and 20. NF-κB consensus oligonucleotide (Promega) was used for gel shift assay.
Gene for the Novel Mouse Cytokine tag7

18635

Tag7 Is a Secreted Protein and Secretion Can Be Activated in Lymphoid Cells—To study Tag7 expression at the protein level, rabbit polyclonal antibodies raised against the E. coli Tag7 recombinant protein were used for Western blotting analysis of VMR-L and CSML-0 cells (Fig. 5A). The cells were separated from the cultivation medium and lysed. Proteins from both cellular lysates and cultivation medium were immunoprecipitated with anti-Tag7 antibodies, separated in polyacrylamide gel, and transferred to membrane. Most of the Tag7 protein was detected in conditioned medium of VMR-L cells (Fig. 5A). A similar pattern of gene expression was observed for another Tag7-expressing cell line, CSML-0 (data not shown). The level observed in the brain, where the distribution of tag7-expressing cells was extremely nonrandom. In the cerebellum, Purkinje cells were specifically labeled. Similarly, only certain layers of neurons were positive in hippocampus (Fig. 3, B and C). Finally, strong expression of the tag7 gene could be detected among the cells filling the space within the intestinal villus (Fig. 3D).

Considering a similarity in distribution of cis-regulatory elements of the tag7 and LT-β genes, we analyzed tag7 expression in some mouse lymphoid cells. Northern blot analysis was performed with total RNA isolated from mouse circulatory monocytes, thymocytes, splenocytes, and resident peritoneal macrophages (Fig. 4). All cells except for monocytes displayed a constitutively high level of tag7 mRNA.

Stimulation with IL-2 or phytohemagglutinin did not lead to a significant increase of the mRNA content in thymocytes and macrophages in the time course studied (data not shown). In mouse splenocytes cultured with LPS, the level of tag7 transcript dropped down within the first hours of activation (Fig. 4). After the first 5 h of LPS treatment, the level of tag7 mRNA decreased twice; thereafter, induction took place, but the increase of the mRNA level was relatively low. The highest observed level of tag7 mRNA was observed after 24 h of stimulation (Fig. 4). However, Northern blot analysis of murine B cell lymphoma cell line WEHI-231 and T cell lymphoma LBRM-33 did not reveal any detectable level of tag7 transcripts (data not shown) and furthermore, no tag7 transcripts were detected in the above cell lines stimulated with phorbol ester, LPS, and calcium ionophore for 20 h (data not shown).

Tag7 transcription in different tumors and its alteration after establishment of tumors as cell lines. Northern hybridization of total RNA isolated from different sources with labeled tag7 cDNA. All lanes used 20 μg of total RNA. Tag7 transcript is indicated by the arrow. Below, hybridization of the same membrane with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown.

Chromosomal Mapping of tag7—To determine chromosomal localization of tag7 in mouse genome, metaphase chromosomes were analyzed by fluorescence in situ hybridization. A total of 80 metaphase cells were analyzed; 62 exhibited specific labeling. This experiment resulted in the specific labeling of the centromeric region of chromosome 7 (data not shown). Measurements of 10 specifically hybridized chromosomes 7 demonstrated that signal is located at a position that is 9% of the distance from the centromere, an area that corresponds to band 7A3.

Transcription of tag7 is specific for lymphoid and some other cells—Northern hybridization was used to estimate the correlation between tag7 mRNA expression and metastatic properties of tumors. However, no correlation was observed upon hybridization with total RNA of CSML-0 and CSML-100 tumors (Fig. 2). Moreover, the expression of the tag7 gene appeared to be specific in this pair for the tumor with a low metastatic capacity, CSML-0. There was also no correlation between metastatic potential and tag7 mRNA expression in other tested murine tumor cell lines (data not shown). The tag7 mRNA level dramatically altered after the establishment of the tumors as cell culture (Fig. 2). In an established cell line from VMR-L tumors, the level of tag7 transcription dropped down, whereas in the CSML-0 cell line, obtained from corresponding tumors, it appeared to be up-regulated. Thus, tag7 in transplanted tumor is regulated by the host factors.

To determine the tissue-specific pattern of tag7 transcription, Northern blot analyses and in situ hybridization were performed. Northern hybridization of several adult mouse tissues (Fig. 3A) revealed the highest level of tag7 transcription in the lungs and spleen, a detectable level in brain and thymus, and no mRNA content or just a very low level in the other tissues tested. However, in situ hybridization performed on sections of selected organs allowed us to detect a high level of tag7 transcripts in certain areas of brain and intestine (Fig. 3, B–E). Only single, randomly distributed cells were labeled in the thymus. In the lungs, the label was concentrated in the intraalveolar space, where, presumably, alveolar macrophages are located (data not shown). Very characteristic pictures were
of tag7 transcription in CSML-0 cell line was higher than in VMR-L cells (Fig. 2); however, the level of protein synthesis remained low, and we were unable to detect Tag7 protein without immunoprecipitation with antibodies. In freshly isolated mouse splenocytes, tag7 mRNA was present at a rather high level, but Tag7 protein was also detected in these cells only after immunoprecipitation with anti-Tag7 antibodies. Mouse splenocytes (Fig. 5B) contained a significant amount of cellular Tag7 protein, and LPS stimulation increased its content 5–7-fold with a maximum at about 24 h. Although during the first hours of activation most of Tag7 protein was found in a splenocyte-associated form, a long exposure of cells to LPS resulted not only in increasing the overall amount of the protein but also in its secretion. To determine the quaternary structure of the protein, cultivation medium from LPS-activated mouse splenocytes was concentrated and applied on Superdex 75HR column. Fractions were collected and concentrated, and Western blot analysis with anti-Tag7 antibodies was performed (Fig. 5C). About half of the total amount of the protein was detected in a 50-kDa fraction; the rest of the protein remained in low molecular weight fractions. This fact allowed us to suggest that secreted native Tag7 protein could also exist as a multimeric complex.

**Secreted Tag7 Induces Cell Death in an Apoptotic Manner—Recombinant E. coli Tag7 protein was isolated as a denatured protein from inclusion bodies. All of our attempts to refold the protein after purification on Ni-NTA column failed. During dialysis, the purified from the E. coli system material irreversibly precipitated. To perform functional studies with Tag7 protein, we constructed cell line constitutively producing Tag7.**

The VMR-0 cell line, which does not express tag7 (Fig. 6A), was stably transfected with the constructions expressing the tag7 gene. For transfections, both pBK-CMV and pM5Gneo eucaryotic expression vectors were used; however, the pM5Gneo vector allowed us to obtain significantly higher level of Tag7 expression. We analyzed 15 G418-resistant clones on the level of Tag7 expression. In most of them, the level of the exogenous tag7 transcription was relatively high, but Tag7...
protein synthesis remained hardly detectable. The clone with the highest level of Tag7 secretion (VMRSX8) (less than 10 ng/ml) was selected for further studies of soluble Tag7 protein. For this, L929 cells were treated with either conditioned supernatant from VMRSX8 cells or from the control mock-transfected VMR-0 cells for different time intervals. It was found that conditioned medium from VMRSX8 cells caused death in target L929 cells. Maximum cytotoxicity was observed at 5 h and did not increase significantly after 24 h incubation (Fig. 6B). The results obtained demonstrated that both TNF and Tag7 killed L929 cells in the presence of actinomycin D. Addition of anti-Tag7 polyclonal antibodies specifically blocked cell death caused by VMRSX8 supernatant and did not affect TNF-induced apoptosis. At the same time, anti-TNF antibodies did not block Tag7-induced cell death. The supernatant from VMR-0 cells did not possess any cytotoxic activity. Human breast adenocarcinoma cell line MCF7 was also susceptible to Tag7 killing (data not shown).

Because the process of apoptosis is known to rapidly induce DNA fragmentation, the ability of the secreted form of Tag7 to trigger apoptosis was examined utilizing a DNA fragmentation assay. Conditioned supernatant of VMRSX8 cells was added to target L929 cells. As controls, we used mock-transfected VMR-0-conditioned medium and recombinant TNF. After a 5-h incubation, fragmented DNA in the cytoplasm was recovered and resolved by agarose gel electrophoresis. Murine L929 cells were triggered to undergo apoptosis by the soluble form of the Tag7 protein and recombinant TNF but not with supernatant from VMR-0 cells as, evidenced from intranucleosomal DNA fragmentation (Fig. 6C).

**NF-κB Accumulation Is Not Activated in Target Cells by Tag7**—TNF has been shown to induce apoptosis and NF-κB activation, two of the most important activities signaled by TNFR-1 (22). We investigated whether Tag7 also activates NF-κB. VMR-0 cells stably transfected with tag7-expressing construct (VMRSX8) did not show changed viability and proliferation. To investigate the NF-κB activation in sensitive to Tag7 cells, L929 cells were treated with either conditioned supernatant from cells transfected with tag7 (VMRSX8) or recombinant human TNF in concentration 10 ng/ml. Nuclear extracts were prepared 2 h later, and reacted with end-labeled NF-κB specific probe and subjected to EMSA (Fig. 7). Tag7 did not induce detectable NF-κB activation in L929 cells, although cytotoxicity of Tag7 was significantly higher than that of TNF during short-term of exposure of the cells to these cytotoxic agents.

**DISCUSSION**

During the last decade, a number of TNF-related cytokines were identified and cloned. Some of them are produced by tumor cells (11, 23). Using the “differential display” technique, we have isolated a novel mouse gene with a remote homology to the TNF family of ligands. It came out first from the genomic organization of the tag7 gene. It is similar to the mouse LT-β three-exon structure and the noncanonical TATA box with multiple transcription initiation sites. The promoter regions of TNF, lymphotoxins, and tag7 genes show similarity in regulatory elements, although their combination is unique for each gene.

The tissue specificity of tag7 expression also resembles that of mouse LT-β expression with quite a few exceptions. In situ hybridization analysis for tag7 on sections of selected organs also demonstrated the pattern of the signal distribution close to LT-β mRNA. The total level of tag7 mRNA in brain was very low; however, specific cell types gave a strong hybridization signal upon in situ hybridization. Specific cortical areas of the brain (the hippocampus and Purkinje cells of the cerebellum) (Fig. 3, B and C) showed a high level of tag7 mRNA content. The same pattern of gene transcription was observed for the mouse LT-β (21) gene. Only minor differences in the patterns of tag7 and LT-β mRNA distribution were observed. Lymphoid and hematopoietic organs contained Tag7-expressing cells, although in the thymus, the level of tag7 transcription was very low. On the other hand, the level of tag7 transcription in spleen was very high, and on sections, tag7 mRNA was detected almost everywhere (data not shown), whereas LT-β mRNA was predominantly expressed in the white pulp. This implies that two genes may still be differentially regulated and activated through different signaling pathways. A significant level of tag7 transcription in lungs could be attributed to the large amount of alveolar macrophages.

The presence in the regulatory region of the tag7 gene of NF-κB binding site supposed very fast response on well-known stimuli, e.g., LPS or phorbol 12-myristate 13-acetate, which could result in increased transcription of the gene. We detected down-regulation of tag7 transcription in lymphocytes at the early stages of activation. A similar type of down-regulation of TNF mRNA expression in macrophages is mediated through the regulation of NF-κB activation (24, 25). The reduction in tag7 transcription indicates that additional nuclear factors may be missing or that silencers may be activated in such a way that transcription of the tag7 gene is prevented. Activation of the tag7 gene expression was relatively low in splenocytes and tumor cell lines expressing the gene: VMR-L and CSML-0. Alteration in the level of tag7 transcription after establishment transplanted tumors as a cell lines points to the participation of host factors in tag7 transcription. It was further supported by the observation that in B and T cell lines, we were unable to detect tag7 mRNA, although its transcription was abundant in freshly isolated splenocytes. However, we can not rule out the possibility that splenocytes became activated due to isolation procedure.

A constitutive level of tag7 expression in the lymphoid and hematopoietic tissues points to a role for this gene in immune system. Furthermore, it is evident that the main step in regulation cascade occurred at the posttranscriptional level. Even insignificant changes in the mRNA production result in a change of overall amount of protein synthesized and its secretion, although constitutive level of Tag7 protein in isolated lymphoid cells is rather high.

We did not find any significant homology for Tag7 with any known proteins. However, similarity in genomic organization and expression pattern with lymphotoxin-β motivated us for
more detailed amino acid sequence analysis. Low homology allowed multiple alignments, but this analysis revealed the existence of five regions of low homology in the extracellular domains of the Tag7 polypeptide and TNF family members (Fig. 8). In the first two regions, the level of identity with other TNF family ligands is relatively high, especially in residues buried in the β-sheet interior (26). The rest of the domains show less homology, but the buried residues are still conservative. Moreover, gel filtration data support the similarity in quaternary structure of Tag7 and TNF family ligands. Like lymphotoxins and TNF itself, native secreted Tag7 was detected as a multimer, and the molecular weight of the complex is unknown. Tag7 forms homo- or heterotrimers is unknown. Tag7 is rich in cysteine residues and, like LT-α, contains 4 methionine residues (27).

The tag7 gene was mapped on mouse chromosome 7. Previously, it was observed that many of TNF-related genes are clustered in the genome. TNF/lymphotoxins genes are linked to one another, but not always to the same chromosome. For example, the tag7 gene does not map on the same chromosome with other TNF ligand family members. Chromosome location offers a possible in vivo role of the tag7 gene. The cyto genetic band 7A3 has been genetically linked with lupus-like nephritis in the MRL and New Zealand hybrid models of systemic lupus erythematosus (31, 32). Although systemic lupus erythematosus is unlikely to involve mutations with severe functional alterations, gene knockout experiments may provide insight into pathogenic processes.

Tag7 released in conditioned medium possesses cytotoxicity and triggers intranucleosomal DNA fragmentation in target cells in the same way as many known members of the TNF family. Fragmentation of DNA is one of the characteristics of apoptosis. We can not exclude possibility that Tag7 acts via binding with known “death domain” receptors, but it is unlikely that TNF receptor is involved in the apoptotic cell death caused by Tag7. Cell lines that naturally produce Tag7 protein, such as cell lines that produce substantial levels of Tag7, would induce NF-κB activation, as observed for TRAIL (TNF-related apoptosis-inducing ligand) receptors (38).

A tumor can produce cytokine; this may have important consequences, which may be direct (promoting or inhibiting tumor growth) or indirect (changing such growth through interactions on the microenvironment). The effect of a single cytokine, however, cannot be readily predicted, because its presence induces other cytokine(s), which can significantly affect the primary action. Recent studies with the tumor cells transformed with tag7 demonstrated the important role of the gene in tumor growth.

Acknowledgments—We thank O. Borodulina and A. Ruzov for technical assistance and I. Korobko for help in manuscript preparation.

REFERENCES

1. Pekarek, L. A., Weichselbaum, R. R., Beckett, M. A., Nachman, J., and Schreiber, H. (1993) Cancer Res. 53, 1978–1981. 2. Kruger-Krasagakes, S., Krasagakis, K., Garbe, C., and Diamantstein, T. (1995) Recent Res. Cancer Res. 139, 155–168. 3. Van Meer, G. (1995) Glia 13, 284–288.

FIG. 8. An amino acid sequence comparison of Tag7 and the TNF family of ligands. The alignment is arranged to show maximum similarity of Tag7 and the TNF-like cytokines that are most close to Tag7. The similarity in the rest of the sequence is much lower than shown here. b indicates residues buried in the β-sheet interior of ligands of the TNF family that are similar for the proteins shown.

Amino Acid Comparison of Tag7 and TNF Family Members

| Tag7 | hTNF | hLT-α | hLT-β | hCD40L | mLTA-β | tag7 |
|------|------|-------|-------|--------|--------|------|
| PVAHV 93 | P-VW-EPY | -- | LGGVFQLEKGRDLSAE | 111 |
| PAALHL 68 | P-WY-HSMY | HCAFQFTQSDLTH | 184 |
| PAALHL 93 | GPLWY-TSVG | FGLVLQRLGERVVY | 222 |
| IAALHI 76 | GLFGVFLQPGASFVGN | 240 |
| PVRVYV 45 | GSLWY-TSVG | FGLGALQRGGERVVN | 184 |
| b b b | b b b | b b b | b b b |

hTNF: 88 kDa, hLT-α: 63 kDa, hLT-β: 68 kDa, hCD40L: 122 kDa, mLTA-β: 154 kDa, tag7: 40 kDa.

2. S. L. Kiselev, O. S. Kustikova, E. V. Korobko, and G. P. Georgiev, manuscript in preparation.
24. Takasuka, N., Tokunaga, T., and Akagawa, K. S. (1991) J. Immunol. 146, 3824–3830
25. Takasuka, N., Matsuura, K., Yamamoto, S., and Akagawa, K. S. (1995) J. Immunol. 154, 4803–4812
26. Smith, C., Farrah, T., and Goodvin, R. G. (1994) Cell 76, 959–962
27. Aggarwal, B. B., Henzel, W. J., Moffat, B., Kohr, W. J., and Harkins, R. N. (1985) J. Biol. Chem. 260, 2334–2339
28. Spies, T., Morton, C. C., Nedospasov, S. A., Fiers, W., Pious, D., and Strominger, J. L., (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 8699–8702
29. Nedospasov, S. A., Hirt, B., Shakhoev, A. N., Dobrynin, V. N., Kawashima, E., Accolla, R. S., and Jongeneel, C. V. (1986) Nucleic Acids Res. 14, 7713–7725
30. Gardner, S. M., Mock, B. A., Hilgers, J., Huppi, K. E., and Roeder, W. D. (1987) J. Immunol. 138, 476–483
31. Kono, D. H., Burlingame, R. W., Owens, D. G., Kuramochi, A., Balderas, R. S., Balomenos, D., and Theofilopoulos, A. N. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 10168–10172
32. Morel, L., Rudolfsky, U. H., Longmate, J. A., Schifffenbauer, J., and Wakeland, E. K. (1994) Immunity 1, 219–229
33. Korobko, E. V., Suschenko, L. P., Prockhorchouk, E. B., Korohko, I. V., Gouchev, V. N., and Kiselev, S. I. Cell Death Differ. in press
34. Chinnaiyan, A. M., O'Rourke, K., Yu, G. L., Lyons, R. H., Garg, M., Duan, D. R., Xing, L., Gentz, R., Ni, J., and Dixit, V. M. (1996) Science 274, 996–992
35. Kitson, J., Raven, T., Jiang, Y. P., Geeddel, D. V., Giles, K. M., Pun, K. T., Grinham, C. J., Brown, R., and Farrow, S. N. (1996) Nature 384, 372–375
36. Marsters, S. A., Sheridan, J. P., Donahue, C. J., Pitti, R. M., Gray, C. L., Goddard, A. D., Bauer, K. D., and Ashkenazi, A. (1996) Curr. Biol. 6, 1669–1676
37. Baldwin, A. S. (1996) Annu. Rev. Immunol. 14, 649–683
38. Pan, G., O'Rourke, K., Chinnaiyan, A. M., Gentz, R., Ebner, R., Ni, J., and Dixit, V. M. (1997) Science 276, 111–113