Bamacan is a chondroitin sulfate proteoglycan that abounds in basement membranes. To gain insights into the bamacan gene regulation and transcriptional control, we examined the genomic organization and identified the promoter region of the mouse bamacan gene. Secondary structure analysis of the protein reveals a sequential organization of three globular regions interconnected by two α-helix coiled-coils. The N- and the C-terminal ends carry a P-loop and a DA box motif that can act cooperatively to bind ATP. These features as well as the high sequence homology with members of the SMC (structural maintenance of chromosome) protein family led us to conclude that bamacan is a member of this protein family. The gene comprises 31 exons and is driven by a promoter that is highly enriched in GC sequences and lacks TATA and CAAT boxes. The promoter is highly functional in transient cell transfection assays, and step-wise 5′ deletions identify a strong enhancer element between −659 and −481 base pairs that includes Jun/Fos proto-oncogene-binding elements. Using backcrossing experiments we mapped the Bam gene to distal chromosome 19, a locus syntenic to human chromosome 10q25. Bamacan is differentially expressed in mouse tissues with the highest levels in testes and brain. Notably, bamacan mRNA levels are low in normal cells and markedly reduced during quiescence but are highly increased when cells resume growth upon serum stimulation. In contrast, in all transformed cells tested, bamacan is constitutively overexpressed, and its levels do not change with cell cycle progression. These results suggest that bamacan is involved in the control of cell growth and transformation.

Proteoglycans are specialized glycoproteins found in all connective tissues and on the surfaces of cells (1). The heterogeneity of proteoglycan structure is a reflection not only of the variation in amino acid sequence of the protein core but also variation in the type and size of their glycosaminoglycan chains (2, 3). There is mounting evidence that the proteoglycan constituents of the extracellular matrix play a crucial role in modulating cell phenotype and growth. This has been particularly well established with regard to the neoplastic process inasmuch as remodeling of the extracellular matrix represents an important hallmark of the disease (4). A large body of literature exists documenting the presence of increased levels of proteoglycans with altered composition in human tumors, primarily those of epithelial origin, such as breast, colon, and lung (5). Furthermore there is convincing evidence that tumor formation is associated with aberrant expression of proteoglycans (6). For instance, altered proteoglycan biosynthesis is a phenotypic trait of neoplastic cells that is maintained at the metastatic site (7, 8) and changes in proteoglycan expression either precede (9) or are induced by malignant transformation (3).

Bamacan is a chondroitin sulfate proteoglycan originally isolated from organ cultures of embryonic parietal yolk sac (Reichert’s membrane) (10, 11). Bamacan has been recently identified as a component of the basement membrane in the Engelbreth-Holm-Swarm tumor matrix (12), the renal mesangial matrix (13) and possibly of the basement membrane of other tissues (14). Its amino acid sequence, deduced from the cloned rat cDNA (15), reveals unique features among proteoglycans such as the presence of two coiled-coil domains and protein motifs potentially implicated in cell adhesion. The attachment of glycosaminoglycan chains is possible at five potential glycanation sites located at both the N- and the C-terminal ends. The functional role of bamacan can be only speculated at present, although developmental studies suggest that bamacan expression is tightly regulated during mammalian embryonic development (16, 17).

In this study, we report the complete characterization and sequencing of the murine bamacan (Bam) cDNA, the genomic organization, and the structural-functional characterization of the promoter. In addition, we mapped the Bam gene to distal chromosome 19. The high conservation of the deduced protein core in comparison with rat, human, and bovine bamacan proteins supports the concept that the protein may play crucial roles in cell biology. The strong sequence homology with the SMC (structural maintenance of chromosome) family of proteins and in particular the similarity to the SMC3 subclass predicts that this proteoglycan may be implicated in chromosome dynamics including chromosome condensation, duplication, and X chromosome dosage (18, 19). The architecture of the bamacan promoter reveals several cis-acting elements capable of recognizing nuclear factor proto-oncogenes and factors involved in cell cycle regulation, whereas the remarkable complexity of the genomic organization raises the possibility that bamacan splicing variants might be expressed. Notably, bamacan is differentially expressed in murine tissues, and its levels are dramatically enhanced in transformed cells. In normal cells, its expression varies as a function of their growth status.
Collectively, these results suggest that bamacan may play a role in cell growth and transformation.

Experimental Procedures

Materials and Cells—All reagents were of molecular biology grade. [α-32P]dATP and [γ-32P]dATP (~3000 Ci/mmol) were obtained from Amersham Pharmacia Biotech. Cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum. The cell lines utilized were: 3T3 Swiss albino mouse fibroblasts that display contact inhibition and serum dependence and a 3T3-L1 clone obtained in our laboratory that has lost sensitivity to contact inhibition; mouse mammary C3 epithelial cells and their malignant counterpart C7 (a gift of J. E. Knepper, Villanova University) derived from normal and neoplastic mammary tissue of MMTV-v-Ha-ras transgenic mice, respectively; a highly metastatic variant of the Lewis lung carcinoma cells (HM-LLC); the immortalized MLE-10 hepatocytes (20); BAM1, an Engelbreth-Holm-Swarm tumor-derived cell line producing basement membrane components (21); J774-A1 murine macrophages; YAC-1, a lymphoma cell line growing in suspension; M2 melanoma; CMT-93 colon carcinoma; and COMMA-D, a mammary tumor cell line.

Identification and Characterization of the cDNA and Genomic Clones—Based on the published sequence for rat bamacan cDNA (15)...
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Identification and Functional Analysis of the Promoter—A segment of DNA 5′ of exon 1 or step-wise deletions were subcloned into a pGL3 luciferase reporter vector (Promega). Specifically, a DraI-BgrI fragment was excised from the genomic EcoRI subclone containing this sequence and digested with the Smal-NcoI sites of the reporter vector. To achieve directional insertion and at the same time convert the BgrI and NcoI ends to compatible 5′ overhangs, the promoter insert and the linearized vector were incubated with T4 DNA polymerase in the presence of dNTPs mixture to achieve partial filling. Further manipulation of the promoter sequence was carried out by taking advantage of the SacI, ApaI, and BulI restriction sites located within the promoter sequence. All the constructs were analyzed by restriction digestion, and the relevant ligation sites were fully sequenced. Transient transfection was performed by a modified calcium phosphate method. Briefly, various constructs were mixed with a solution containing calcium-phosphate Maximizer (Stratagene) in 2 M CaCl₂ and added dropwise to a 2 × Hanks phosphate buffer. After careful mixing, the solution was added to 60 mm glass coverslips and the incubation was continued for 8 h. The transfection mixture was then removed, and the cells were fed with regular medium. After 24 h the luciferase activity was assayed using the dual luciferase reporter assay System (Promega). As an internal control we used an empty firefly luciferase vector and a SV40-pGL3 vector in which the firefly luciferase expression is driven by the early SV40 promoter. The cytomegalovirus Revilla luciferase vector was used as internal control to normalize for the efficiency of the transient transfection. All the samples were analyzed in quadruplicate, and two sets of experiments were carried out.

Primer Extension, Northern Blotting, and Chromosomal Mapping—Total RNA was isolated using the Tri-Reagent solution from subconfluent cells and stored in 70% ethanol at −20 °C until used. Synthetic oligonucleotides (21 bp) antisense to complementary cDNA spanning a 21-bp region 3′ to the ATG site, were end-labeled with [32P]ATP using 10 units of T4 polynucleotide kinase (Promega). Following incubation at 37 °C for 10 min, the enzyme was heat inactivated at 90 °C for 15 min, and the radiolabeled primers were purified by chromatography on a prepacked RNAse-free Sephadex G-25 column. Specific activity of the labeled probe was >10⁶ cpm/mmol. For primer extension, 1 μg of the labeled probe was incubated with 10 μg of total RNA and heated at 85 °C for 5 min, and [32P]-labeled DNA-RNA annealing was allowed to proceed at 50 °C for 1 h. Primer extension was completed by addition of reverse transcriptase (Promega) in the presence of dNTPs at 42 °C for 30 min and stopped by the addition of 70% ethanol. The precipitated DNA was dissolved in 30% formamide and loaded onto a 6% polyacrylamide sequencing gel. Northern blotting with probes spanning various regions of the bamacan cDNA was performed as described before (22). Additional details are provided in the text and legends to figures. For chromosomal mapping, we used an interspecific backcross of (AEJ/Gn-a × Mus spretus) × AEJ/Gn-a × Mus spretus (23). Segregation patterns for the loci were determined from random subsets of 195 N2 progeny. The Bam locus was identified using two distinct probes, P144 and P164, from the murine bamacan cDNA. Primer pairs identifying the D19Mit1 and D19Mit75 loci were purchased from Research Genetics, Inc. (Huntsville, AL). PCR conditions were 94 °C for 4 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 45 s, ending with a single cycle at 72 °C for 7 min. PCR products were analyzed on 2.5% agarose gels.

RESULTS AND DISCUSSION
cDNA and Genomic Cloning of Mouse Bamacan—Based on the published sequence for rat bamacan cDNA (15), a computer search of the EST Data Base for homologous mouse and human sequences was performed. This search led to the identification of 20 potential clones for which cDNA sequences originating from 6 mouse cDNA libraries and 16 potential clones for human bamacan from 3 human cDNA libraries. Seven mouse clones were fully sequenced providing more than 95% of the mouse bamacan cDNA (Fig. 1A). The leading sequence of the 5′-untranslated region and an internal segment (bp 1469–1526 of the cDNA) were not present in the clones considered; these se-

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1 The abbreviations used are: EST, expressed sequence tag; kb, kilo-base pair(s); bp, base pair(s); PCR, polymerase chain reaction.
sequences were determined by analyzing genomic clones. To this end, a 129/SvJ mouse genomic library was screened using zP-labeled cDNA probes P144, P164, and P240, covering respectively 1404 bp of the 5' region, 857 bp of the middle region, and 843 bp of the 3' end. In addition, a PCR-generated probe spanning 330 bp at the 5' end of cDNA clone AA144255 was also used. With this strategy we isolated a series of overlapping genomic clones encompassing the entire coding sequence and further extending ~7 kb upstream of the putative transcription start site. Clone Xba-6.2, derived from phase 3/164, encompassed the sequence that was missing from the EST clones, thereby allowing the determination of the complete mouse cDNA. The strategy utilized for the identification of this clone is illustrated in Fig. 1B. To identify the size of the bamacan transcript, murine RNA was hybridized with zP-labeled probes spanning two separate areas of the bamacan cDNA. The results revealed a single bamacan transcript of ~4.2 kb (Fig. 1C) using the two cDNA probes.

While this work was underway, Gupta et al. (24) reported the cloning of a mouse protein denoted Mmip1 with cDNA sequence identical to that of mouse bamacan between bp 1523 and 4138 (Fig. 1A). The size of the Mmip1 transcript corresponded to that of mouse and rat bamacan (i.e., ~4 kb). Mmip1 cDNA coded for a protein of 485 amino acids whose sequence matches that of mouse bamacan between residue Met-537 and residue Met-1018. This short form of bamacan cDNA was generated by the presence of additional G and A intercalating the residue Met-6.2, derived from phage 3/164, encompassed the sequence that was missing from the EST clones, thereby corroborating the correctness of our sequencing. Fourth, both the cDNA and deduced amino acid sequences of mouse bamacan matched those of rat bamacan with a high degree of homology. Fifth, the open reading frames are of identical size. Finally, the mouse and the rat bamacan sequences are highly homologous (~98%) to HCAP, a human protein that is associated with chromosomes (henceforth identified as human bamacan) (26). Based on this compelling evidence, we conclude that Mmip1 is either the product of an as yet unidentified mRNA editing process or, more likely, a truncated cDNA with scrambled ends.

The Murine Bamacan Protein—Compared with the published sequence of rat bamacan (15), the mouse protein (Fig. 2) extends for an additional 32 amino acids at the C terminus. The early termination of the rat bamacan coding sequence is due to the presence of a stop codon located at bp 3668. In contrast, mouse and human bamacan cDNAs are of the same length and encode for proteins of identical amino acid number. Bamacan, unlike other proteoglycan core proteins, displays a high degree of sequence conservation in the three species thus far examined. Protein conservation is at the ~98% level between mouse, rat, and human. In particular, mouse bamacan differs from the rat protein at residue 517 (Asp to Glu), 735 (Ile to Thr), 786 (Leu to Pro), 810 (Glu to Lys), 865 (Glu to Gln), and 1051 (Gly to Ala). The difference between mouse and human bamacan is even more contained, consisting of a conservative Glu to Asp substitution at residue 517. Primary structure analysis fails to reveal a signal peptide sequence. Potential glycanation sites (Ser-Gly)
are present at residues 36, 249, 1073, 1081, and 1116. The sequences ETSGE and GEGSGE beginning at residues 247 and 1071, respectively, are also present in the cell surface heparan sulfate proteoglycan syndecan-1 (27) where they act as the attachment sites for chondroitin sulfate. The latter motif is also found in the chondroitin sulfate proteoglycan versican (28). The ability of some of these sequences to act as functional glycanation sites has been directly demonstrated by expression in COS-7 cells of a fusion protein composed of bamacan amino acid 1029–1128 sequence fused to the IgG-binding portion of protein A (15). Metabolic labeling of the transfected cells gave rise to a product that migrated as a broad smear on SDS-polyacrylamide gel electrophoresis in untreated or heparinase III-treated samples but as a discrete product after chondroitinase ABC digestion, consistent with the fusion protein being secreted as a chondroitin sulfate proteoglycan (15).

Other potentially relevant motifs are found at position 552 (VTxG), which has been implicated in mediating cell adhesion and an LRE motif at residue 850. Further, a leucine zipper DNA-binding domain can be identified extending from residue 800 to 821 and is harbored in an amphipathic α-helical region (24). Finally, the sequences NGSGKSN (P-loop motif) and LS-GGQ X_2 DEX_LD (DA box motif) starting at residues 32 and 1114, respectively, are highly conserved in SMC proteins and are potential sites for ATP binding (19, 29).

Organization of the Bamacan Gene vis à vis the Protein Modules—Sequence of the genomic clones spanning the Bam locus identified 31 exons (Table I) contained within ~45 kb of genomic DNA (Fig. 3). Exons range between 39 bp (exon 3) and 440 bp (exon 31) in size. Exons 14–21 are located within large intronic sequences. The rat bamacan protein has been postulated to be organized into five structural domains (15). The information we have assembled on the genomic organization allowed the matching of these domains to specific groups of exons of the bamacan gene. Domain I is predicted to assume a globular conformation and is coded by exons 1–8. The highly conserved NGSGKSN P-loop motif is encoded by a single exon (exon 3). Exons 9–17 code for a protein domain that can assume α-helix coiled-coil conformation. Exon 10 contains the ETSGE consensus sequence for glycayation. The third structural domain of the protein is coded by exons 18–20. This short connecting domain can assume a globular conformation. This domain is followed by a fourth domain that can assume an α-helix coiled-coil conformation similar to that of domain II. This domain is encoded by exons 21–27 and harbors the leucine zipper DNA-binding domain (exons 23 and 24). Domain V may assume a globular conformation and is coded by the exons 28–31. This domain contains the GEGSGE consensus sequence for glycayation, which is coded by exon 28. The DA box motif is coded by exon 29.

Domains I, III, and IV share a high degree of sequence homology with members of the SMC protein family, whereas domains II and IV are homologous to the myosin heavy chain. The sequential structural organization and protein homology as well as the presence of a P-loop and DA box motifs, respectively, at the N- and the C-terminal ends of the protein identify bamacan as a member of the SMC protein family. Recently the protein sequence of bovine SMC3 has been made available (GenBank™ accession number AF072713). The protein has the same degree of homology to mouse bamacan as the rat and the human proteins. We therefore assign bamacan to the SMC3 subfamily. The other members of this family are located intracellularly and are implicated in the condensation of chromatin and in gene dosage mechanism (30). These proteins can directly interact with DNA. The P-loop and the DA box are believed to act cooperatively to bind ATP. When this occurs it triggers the structural alteration of the molecule leading to its contraction and the condensation of the bound DNA to form chromatin.

Extracellular versus Intracellular Species—Bamacan, unlike the other members of the SMC protein family, has been detected both extracellularly and intracellularly within the nucleus. The lack of a signal peptide is at odds with the observation that all the extracellular proteoglycans thus far identified have a well conserved signal peptide. On the other hand, the structural motif analysis of the bamacan amino acid sequence has further revealed that the protein harbors a “bipartite” nuclear localization motif. This motif (K Kel K), located at residue 395 in exon 15, has been shown to signal transport to the nucleus in other proteins (31). Although these results do not exclude the possibility that bamacan can be secreted, they do corroborate the observation that this protein may have primarily a nuclear localization. An intriguing possibility is that the glycayation of bamacan may mediate its extracellular transfer. Perhaps part of the newly translated protein may escape its nuclear fate and be post-translationally modified to become a proteoglycan. Notably, other chromatin-associated proteins have been identified extracellularly despite their lacking a signal peptide. The most remarkable case is that of histone H1, which can act as cell surface receptor for thyroglobulin and has been shown to be secreted and act as a potent growth factor (32). Proteoglycans can also play an important functional role within the nucleus. For example glypican and biglycan, a cell surface proteoglycan and an extracellular matrix proteoglycan, respectively, have been immunologically detected in the nuclei.
of neuronal cells and are thought to play a specific function during the cell cycle (33). Both display chromosomal binding, but in addition glypican displays dynamic properties moving from the cytoplasmic compartment to the nucleus during mitosis. The core proteins of both proteoglycans harbor a nuclear localization signal that appears to be functional inasmuch as its mutation leads to ablation of their ability to move to the nucleus during mitosis.

**Structural and Functional Characterization of the Bamacan Promoter—Sequence analysis of the 5′-flanking region of the Bam gene** (Fig. 4A) revealed the presence of several cis-acting factor-binding motifs involved in growth control and cytokine stimulation such as: IRF1, a primary target of signal transcription induced by interferon-β and interleukin-6; NF-I, which binds a nuclear factor induced by TGF-β; two GR motifs elements recognizing the glucocorticoid receptor; and a E2F-1 recognizing a factor involved in cell cycle regulation (34). The presence of E2F-1 cis-acting elements is noteworthy insofar as it has not yet been identified in the promoter region of any other proteoglycan thus far investigated (35). Members of the E2F transcription factors regulate the expression of a number of genes important in cell proliferation, particularly those involved in progression through the G1 phase and into the S phase of the cell cycle. The activity of E2F factors is regulated through association with the retinoblastoma tumor suppressor protein. The presence of cis-acting elements for proto-oncogene
The Murine Bamacan Gene

Fig. 5. Deletion construct of the bamacan gene promoter and summary of the luciferase activity. A, schematic representation of the 5’ step-wise deletion constructs used to test the functional activity of the bamacan promoter in transient transfection assays and relative luciferase activity of each construct are shown. Numbers to the left of each construct indicate the 5’ end of the promoter fragment relative to the major transcription start site (+1) of the bamacan gene. B, summary of luciferase expression assays of bamacan gene promoter and various 5’ deletion constructs. C7 mammary carcinoma cells were co-transfected with the various bamacan promoter-firefly luciferase constructs and pRL-CMV plasmid carrying the Renilla luciferase gene. Firefly luciferase activity was assayed as described in the text and is expressed as a percentage relative to the maximum firefly luciferase activity produced by the −659-bp bamacan construct. Value were normalized for transfection efficiency based on the Renilla luciferase value recorded in the same cells. The values represent the normalized means ± S.D. of four different experiments.

acting as nuclear transcription factors is noteworthy in relation to the possible involvement of bamacan in cell transformation. In particular, a v-fos/jun/c-fos binding motif is located 0.6 kb upstream of the major transcription start site (see below).

To establish the transcription start site(s), primer extension was performed using total RNA from 3T3-L1 fibroblasts, HMLLC lung carcinoma cells, and M2 melanoma cells. The extension products were analyzed on a 6% denaturing polyacrylamide sequencing gel using a ladder of labeled DNA standards. Multiple transcription start sites were identified using various mRNAs (Fig. 4B). 3T3-L1 RNA generated several bands that predict the location of the putative transcriptional start sites to be located between −285 and −59 bp upstream of the ATG. RNA from HM-LLC and M2 cells generated fewer extension products. The larger of these product was the same for both cells and would be generated by a transcription start site located 95 bp upstream of the ATG site. Because the same fragment was also detected as the major extension product in 3T3-L1, we use this as the primary transcription start site. Multiple transcription start sites are frequently observed in genes that lack TATA and CAAT boxes and are GC-rich, as in the bamacan gene.

Next, we sought to determine whether this region could act as a functional promoter in transient cell transfection assays. For this purpose, a 752-bp genomic fragment flanking the 5’ region of the Bam gene was cloned upstream of a firefly luciferase reporter gene in a pGL3 vector. This construct was transfected into six different cells with diverse histogenetic backgrounds along with a Renilla luciferase reporter gene driven by a cytomegalovirus promoter to normalize for transfection efficiency. In all the transformed cell lines, the bamacan promoter displayed high activity when compared with the activity detected in normal, nontransformed cells (Fig. 4C). In particular, the highest promoter activity was detected in C7 mammary carcinoma cells. Expression in hepatoma MLE-10 cells and in melanoma M2 cells was approximately one-fourth that detected in C7 cells. By comparison the promoter activity in normal mammary cells (C3) and fibroblasts (3T3) was only small percentage of the value detected in C7 cells. The bamacan promoter activity in CMT-93 rectal carcinoma cells was similarly low, suggesting that bamacan expression may be regulated differentially in neoplastic cells.

Further experiments using the most responsive C7 mammary carcinoma cells were carried out to investigate the functional role of the cis-acting elements. For this purpose, step-wise deletions of the bamacan promoter were generated (Fig. 5A). Deletion of the distal 178 bp of the bamacan promoter, which harbor two GR and one AP-1 motif, caused a sharp decrease in functional promoter activity (Fig. 5B). Recently an interaction between AP-1/jun/fos proto-oncogenes and the glucocorticoid receptor has been established and may be responsible for the coordination of the gene responses initiated by glucocorticoids (36). The relative proximity of these two cis-acting elements suggests that these region may act as a functional domain. Shortening of the upstream sequence from −489 to −70 did not result in a major change in the promoter activity in C7 cells. In contrast, ablation of the bamacan promoter activity was observed after the deletion of a short 33-bp sequence harboring a UCRBP binding motif, a transcription factor that binds the regulatory regions of many viral and cellular genes.

Mapping of the Mouse Bamacan Gene to Distal Chromosome 19—To identify restriction fragment length polymorphisms useful for mapping the Bam locus, genomic DNA from the progenitor strains of the interspecific backcross, AEJ/Gn and M. spretus, were digested with 14 restriction endonucleases, and individual digests were analyzed by Southern blot hybridization using the 5’ and 3’ probes for the Bam gene independently. The restriction fragment length polymorphisms used to detect the Bam locus with the P144 probe and the sizes of the EcoRV restriction fragments that distinguished the AEJ/Gn and M. spretus alleles are 10 and 5.9 kb versus >23.1 and 20 kb, respectively. The restriction fragment length polymorphisms used to detect the Bam locus with the P164 probe and the sizes of the PvuII restriction fragments that distinguished the AEJ/Gn and M. spretus alleles are 2.4 and 1.8 kb versus 2.3 and 2.0 kb, respectively. No differences in the segregation patterns for the P144 and P164 probes were detected among...
on the proximal and distal sides of D19Mit75 with respect to other markers are Buchberg and L. D. Siracusa, unpublished data). Loci mapped in hu-

mances centromere -loci were used to align the maps. Our data establish the order of the loci the consensus linkage map of mouse chromosome 19 (41). The dotted cross (described in the text) with the distance between loci given in centimorgans. The chromosome on the right shows the loci typed in the interspecific backcross (described in the text) with the distance between loci given in centimorgans. The chromosome on the right shows a partial version of the consensus linkage map of mouse chromosome 19 (41). The dotted lines between chromosomes indicate that the D19Mit75 and D19Mit1 loci were used to align the maps. Our data establish the order of the loci as centromere - D19Mit75 - Bam - D19Mit1 - telomere based on mapping with respect to other D19Mit markers that were known to reside on the proximal and distal sides of D19Mit75 and D19Mit1 (A. M. Buchberg and L. D. Siracusa, unpublished data). Loci mapped in humans are underlined; locations of genes in the human genome are shown between the chromosomes.

127 N2 progeny (Fig. 6), consistent with the possibility that these two probes represent the 5’ and 3’ ends of a single gene. The segregation patterns obtained for the restriction fragments were compared with markers that scanned the mouse genome, and linkage was found to mouse chromosome 19. The order of the loci and the ratio of the number of recombinants to the total number of N2 offspring is shown in Fig. 6A. The genetic distance between D19Mit75 and P1444, P164, and between P144, P164 and D19Mit1 is 0.8 ± 0.8 centimorgans (Fig. 6B). This location places the Bam gene in close proximity to the Mxi1 gene, a helix-loop-helix leucine zipper that forms heterodimers with Mmi1 and is implicated in the transcriptional regulation of c-Myc (37). These linkage data suggest that the human homolog of the Bam gene most likely resides on human chromosome 10q25.

Bamacan Is Differentially Expressed in Mouse Tissues—To assess the levels of bamacan expression in different organs, bamacan mRNA steady state levels were determined by Northern blotting. A single band migrating slightly faster than the ribosomal 28 S band was detected in all the tissues with an estimated size of ~4.2 kb (Fig. 7A). When normalized to total RNA, the highest steady state levels were detected in the testes and brain (Fig. 7B). Lower levels ranging between 32% and 62% of those detected in the testes were found in muscle, heart, lung, kidney, colon, and thymus. Much lower levels were detectable in parenchymal organs such as spleen, kidney, and liver. In the latter, the bamacan mRNA level was less than 5% that of testes. The same results were obtained using probes spanning bp 60–1464 (P144) or bp 2802–3645 (P240) (data not shown), thus confirming that bamacan is encoded by a single major transcript.

Bamacan Is Abnormally Expressed in Transformed Cells and Tumor Tissues—First, we investigated two sets of cell lines, each composed of the normal and the corresponding transformed counterpart: (i) 3T3 Swiss albino mouse fibroblasts that maintain contact inhibition and are serum-dependent for growth and a 3T3-L1-derived clone that has lost contact inhibition and (ii) mouse mammary C3 epithelial cells and the malignant counterpart C7 cell lines, obtained from normal and neoplastic mammary tissue of MMTV/n-Ha-ras transgenic mice, respectively. A significantly higher (2–4-fold) expression of bamacan mRNA was consistently found in the transformed cell lines (Fig. 8A). To compare the amount of bamacan transcripts synthesized by other tumorigenic cell lines with that of the two transformed cell lines examined above, a panel of mouse transformed cell lines of different origins was investigated under the same standardized condition of growth (Fig.
sented in the matching panels. The human probe P554, a PCR-gener-
comparison, the ethydium bromide staining of ribosomal RNA is pre-

Fig. 8. Abnormal expression of bamacan in transformed cells and neoplastic tissues. A, hybridization autoradiograph of RNA from normal (3T3) and transformed (3T3-L1) mouse fibroblasts, and normal (C3) and malignant (C7) mammary cells. Cells were grown in the presence of 10% serum and harvested upon reaching confluence. Total RNA was extracted with TRI reagent, and RNA was separated by electrophoresis, transferred to nitrocellulose membrane, and hybridized under stringent conditions using bamacan probe P144. B, hybridization autoradiograph of a series of murine tumorigenic cells as indicated. C, autoradiograph of the Northern blot of mouse RNA derived from a normal thymus (lanes 1) and three T-cell lymphomas (lanes 2–4) spontaneously arising from p53/decorin double knockout mice (38). Total RNA was extracted from freshly excised tissue and analyzed by Northern blot hybridization using mouse bamacan cDNA probe P144. For loading comparison, the ethydium bromide staining of ribosomal RNA is presented in the matching panels. D, Northern blot of four sets of normal (N) and tumor (T) samples from subjects with malignant fibrous histiocytoma (lanes 1 and 2) or colon carcinoma (lanes 3–8). For loading comparison, the ethydium bromide staining of ribosomal RNA is presented in the matching panels. The human probe P554, a PCR-generated probe of the human EST AA554698 spanning bp 567–1324, was used.

8B). Several interesting observations were made: (i) a single transcript of ~4.2 kb was detected in all the samples, (ii) the levels of bamacan expression were variable among the cell lines examined, (iii) the highest expression of bamacan was detected in 3T3-L1 transformed cells, in a highly metastatic variant of Lewis Lung Carcinoma cells, and in the mammary carcinoma C7 cells, (iv) lymphoma cells YAC-1 had the lowest bamacan level, and (v) the immortalized hepatic cells MLE-10 (20) expressed significant amounts of bamacan in contrast to the very low level displayed by hepatocytes.

To further assess whether bamacan expression was altered in neoplastic tissues, samples from tumor and unaffected areas of the same organ were analyzed by Northern blot hybridization. The results from spontaneously arising thymic lymphoma cells from p53<sup>−/−</sup> mice, decorin<sup>−/−</sup> double knockout animals (38), and a thymus from a control wild-type animal are illustrated in Fig. 8C. In all the samples, the hybridization signal of bamacan mRNA in tumor tissues, when normalized on total RNA, was consistently higher (2–5-fold) than in normal tissues.

To corroborate the observation of increased bamacan expression in neoplastic tissue, we analyzed bamacan expression in normal human tissues and neoplastic specimens from four cancer patients with malignant fibrous histiocytoma or colon carcinoma. In all the sets of samples, neoplastic tissue consis-

tently displayed higher bamacan RNA levels (Fig. 8D). Thus, bamacan expression is increased in both transformed cell lines and tumor tissues, suggesting a role for bamacan in tumorigenesis.

Dynamics of Bamacan Expression—Because of the link between bamacan expression and transformation we sought to investigate the dynamics of bamacan expression under stimulation by serum. To this end, we synchronized 3T3 and 3T3-L1 fibroblasts by incubating them for 3 days in serum-free medium to attain quiescence. The medium was then supplemented with 10% serum, and the bamacan mRNA levels were quantified at different time points thereafter. Striking differences were observed between the normal and the corresponding transformed cell lines (Fig. 9). Within 30 min after the addition of serum, bamacan expression in the untransformed cells began to increase, and at 60 min it reached a level approximately two times higher than that of the cells kept in serum-free medium. Subsequently, bamacan mRNA began to decrease, reaching base-line values by 2 h, and then began to increase again (not shown). This clearly biphasic behavior in bamacan mRNA level in response to growth stimulation was not observed in neoplastic cells. In this case, the addition of serum did not cause major alteration in bamacan mRNA levels, which remained essentially unchanged for up to 12 h. It should be noted, however, that the absolute levels of bamacan mRNA was 2–3-fold higher in the transformed 3T3-L1 fibroblasts. Thus, it appears that bamacan is constitutively overexpressed in transformed cells. Virtually similar results were obtained when normal and SV40-transformed human skin fibroblasts cells were investigated (not shown). Collectively, these data suggest that bamacan may be part of a set of early responsive genes whose expression is activated upon entry of the cells into the cell cycle. A similar phenomenon has been reported for the transient expression of the proto-oncogenes c-myc and jun-fos (39) and is regarded to have functional significance in initiating a signal cascade leading to activation of genes involved in cell growth.

Conclusions—In this investigation we have cloned and se-
quenced the entire mouse bamacan gene and investigated its promoter activity, its expression in cells and tissues, and its aberrant levels in transformed cells. The fact that bamacan belongs to the SMC3 family of genes (30), which are implicated in gene dosage and DNA repair, suggests that this proteoglycan is destined primarily to the nucleus. More research needs to be done to establish with certainty whether this gene product can be secreted under special circumstances because the high conservation across species puts into doubt the specificity of the cytoplasmic bamacan. The study of the genomic organization reveals that bamacan is a modular protein in which the salient structural motifs are encoded by separate exons. The presence in the promoter of cis-acting protein-binding motifs for oncogenes as well as the constitutive elevation of bamacan mRNA levels in transformed cells and tumor tissues suggest that the expression of this gene may play a role in transformation. Because of the virtual complete conservation of the protein among all the mammal species thus far investigated, it will be of interest to examine whether mutations or deletions of this gene may contribute to the transformed phenotype.

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REFERENCES


The Murine Bamacan Gene 17393
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Giancarlo Ghiselli, Linda D. Siracusa and Renato V. Iozzo

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