mately 15°C below the estimated melting temperature in 3× SSC, 0.1% SDS for 30 min. Final washing temperatures ranged from 54° to 61°C. The filters were then exposed to film at −70°C for 2 to 6 hours. In each case, examples of the hybridizing clones were plaque-purified and sequenced as described in (*11*).
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# In Vivo Footprinting of MHC Class II Genes: Bare Promoters in the Bare Lymphocyte Syndrome

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**Major histocompatibility complex (MHC) class II genes are coordinately regulated and show tissue-specific expression. With the use of in vivo footprinting, common promoter sites in these genes were found to be occupied only in cells that expressed the genes, in spite of the presence of the promoter binding proteins. In vivo analysis of mutant cell lines that exhibited coordinate loss of class II MHC expression, including several from individuals with bare lymphocyte syndrome, revealed two in vivo phenotypes. One suggests a defect in gene activation, whereas the other suggests a defect in promoter accessibility.**

CLASS II MHC GENES CONSTITUTE A multigene family and encode the α and β chains of a group of cell surface heterodimeric glycoproteins that are critical for normal T lymphocyte–mediated immune responses. B lymphocytes constitutively express class II genes, whereas other cell types can be induced to express them (*1*). Class II genes are thought to be coordinately regulated, which would be consistent with the combined loss of expression of all class II genes in individuals with type II bare lymphocyte syndrome (BLS) (*2*) and in experimentally derived mutant B cell lines (*3*). The defect in BLS and these mutant cell lines has been attributed to a trans-acting factor, because the BLS trait and the MHC locus segregate independently (*4*) and class II gene expression can be restored by fusion to a class II MHC–positive cell (*5*). Several complementation groups have been defined by fusion studies (*6*), suggesting that class II gene regulation involves multiple factors.

Attempts to determine the mechanism underlying coordinate regulation have focused on the promoter region of class II genes, which contains motifs conserved in all these genes. This region contains critical cis regulatory elements and confers tissue-specific expression on linked genes in transient transfection assays (*7–9*). Electromobility shift assays have identified factors that are capable of recognizing promoter elements in vitro (*8–11*); these binding activities are present in nuclear extracts of both class II MHC–positive and class II MHC–negative cells, and certain factors have different affinities for the various class II genes in vitro (*11, 12*).

To study the role of the class II gene promoter region in the tissue-specific and coordinate expression of these genes and to gain insight into the BLS defect, we have performed an in vivo genomic footprinting analysis of multiple class II genes by the ligation-mediated polymerase chain reaction (LMPCR), which allows a direct examination of the promoter region (*13*). Because the class II genes are highly polymorphic, we chose to first examine murine genes in cell lines from inbred mice of the H-$2^d$ haplotype, for which the promoter sequences of the four class II genes, $E_\beta$, $E_\alpha$, $A_\beta$, and $A_\alpha$, are available.

We first examined the promoter region of the murine $E_\beta$ gene in the class II MHC–positive B lymphoma line M12.4.1 and in the class II MHC–negative fibroblast line BALB/3T3 (Fig. 1A). The $E_\beta$ gene in M12.4.1 cells displayed protected guanine residues on both the coding and noncoding strands in the conserved X and Y boxes. The Y box contains a reverse CCAAT box, and the two guanines on the coding strand were protected. The X box is located a conserved distance upstream of the Y box and had protected residues both within the X box proper, referred to as $X_1$, and in the adjacent site $X_2$, which overlaps the 3′ end of the X box and the 5′ end of the interspace between X and Y (*1*). A guanine residue at position −120 on the coding strand was neither protected nor hypersensitive and lies between the protected residues of the $X_1$ and $X_2$ sites, demonstrating that these elements are separable. A fourth conserved motif has recently been defined and is called the S box (*1*). No protected residues were observed in the S motif or the surrounding region upstream of the X box, which has variably been called W, Z, or H, and which we will refer to as the S region. A second B lymphoma line, A20, displayed an identical in vivo footprinting pattern to M12.4.1 (*14*). In contrast, no protected or hypersensitive residues were observed in the $E_\beta$ gene from BALB/3T3 fibroblasts on either the coding or noncoding strands in any of the conserved regions.

The footprint patterns of the three other murine class II gene promoters in M12.4.1 cells on both the coding and noncoding strands were very similar to those of $E_\beta$ in the same cells (Fig. 1, B, C, and D). As with $E_\beta$, BALB/3T3 fibroblasts showed no protection of these genes at any of the sites contacted in M12.4.1 cells, although certain hypersensitivities were observed. $A_\beta$ had hypersensitive sites in the $X_1$ site on the

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coding strand and downstream of the Y box on the noncoding strand in BALB/3T3 and M12.4.1. The latter hypersensitive site lies within a purine-rich stretch termed a PU box, which is found in other non–class II genes and therefore may not represent a class II gene–specific motif. The $A_\alpha$ coding and noncoding strands and the $E_\alpha$ noncoding strand showed numerous adenine hypersensitivities in BALB/3T3 cells that were not present in M12.4.1. These hypersensitivities were not associated with corresponding protected regions; they probably do not indicate protein binding, but rather suggest that the conformation of these genes may differ between BALB/3T3 and M12.4.1 cells (*13*).

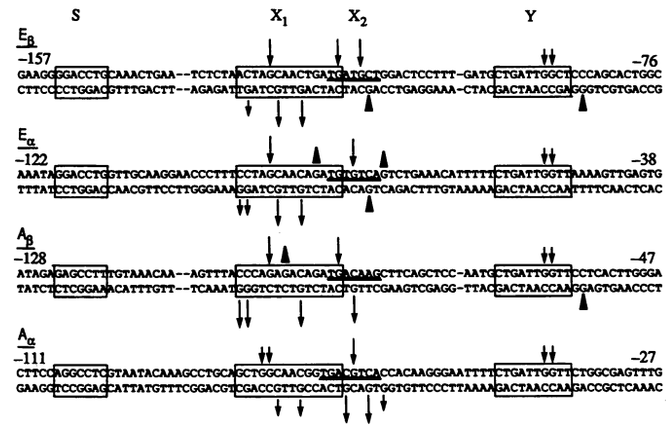


**Fig. 2.** Summary of in vivo footprinting of murine class II MHC gene promoters in M12.4.1 cells. Residues of the murine class II gene promoters that display protection (long arrows), partial protection (short arrows), or hypersensitivity (arrowheads) as detected by in vivo footprinting are shown. The conserved S, $X_1$, and Y motifs are boxed; the $X_2$ site is underlined. Numbers to the left and right of the sequences refer to the first and last nucleotides shown, respectively, and are relative to the transcription start site. Hyphens represent gaps in sequences inserted for best alignment of conserved sequences. All guanine residues of the four class II genes were represented in the primer extension products of the LMPCR reactions except as noted (*26*).

The M12.4.1 data are summarized in Fig. 2 and show that multiple common promoter binding sites are used in all four murine class II genes. These findings are consistent with mutagenesis studies that demonstrated the functional importance of the Y, $X_1$, and $X_2$ sites in transient transfections (*9, 10, 15*). Another conserved element, the S box, was not found to be protected in vivo, although transfection studies have found that deletion of, or mutation within, this region reduces constitutive B cell expression (*8–10*). Aside from experimental differences relating to the transient transfection technique, this region may function other than in the direct binding of a protein, or, alternatively, a protein binding in this region may not contact any guanine residues in the major groove and so would not be detectable in our assay.

The class II MHC–negative fibroblast line BALB/3T3 had no protected sites in vivo at any of the conserved elements of the class II gene promoters, suggesting these elements contribute to the tissue-specific expression of these genes, which is consistent with the tissue-specific expression of class II gene promoter constructs in transient transfections (*7, 8*). However, binding activities directed toward the conserved promoter elements can be detected in vitro in nuclear extracts of BALB/3T3 cells (*14*) and other class II MHC–negative cells (*8, 11*). Thus, for this fibroblast line, class II MHC–negative status is associated with unoccupied class II gene promoters. This finding is similar to results from other genes examined in vivo, including tyrosine aminotransferase (*16*) and muscle creatine kinase (*13*). The lack of class II gene promoter occupancy in BALB/3T3 cells may result from changes in the DNA itself—such as methylation (*16*) or an altered chromatin structure—that affect promoter accessibility (*17*) or from modifications or differences in promoter binding proteins that preclude binding in vivo.

A hallmark of class II MHC–negative regulatory mutants is the combined loss of expression of all class II genes, and so we therefore examined the equivalent promoter region in both normal and mutant human B cell lines. Ramia, Nacera, and BCH are Epstein-Barr virus–transformed cell lines derived from individuals with BLS. BG is a normal-sibling control cell line for BCH. RJ2.2.5 and 6.1.6 are experimentally derived from the parental lymphoma lines Raji and T5.1, respectively.

We examined the $DR_\alpha$ gene because it is the most nonpolymorphic of the human

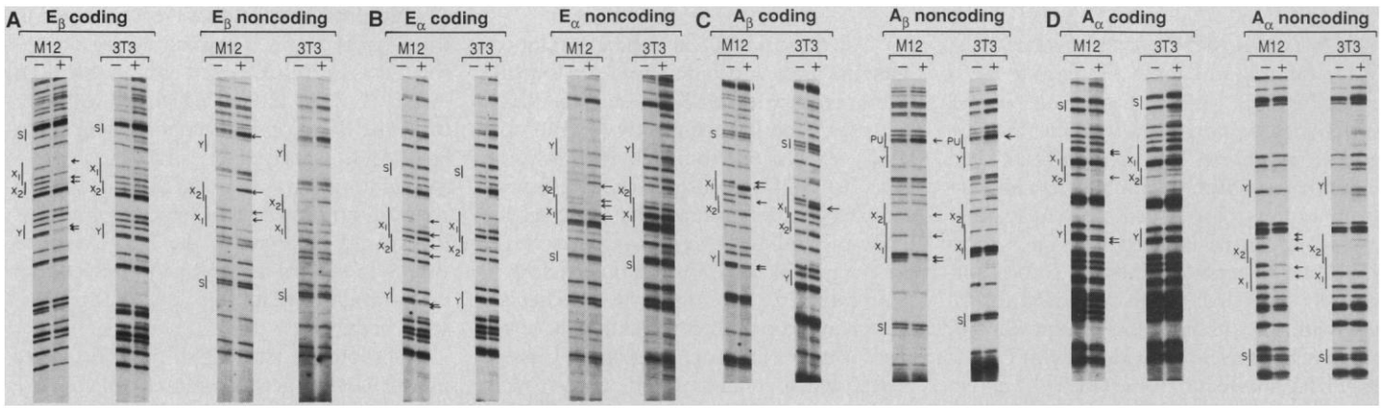


**Fig. 1.** In vivo footprinting of the murine class II MHC gene promoters in a class II MHC–positive B cell line (M12.4.1) and a class II MHC–negative fibroblast line (BALB/3T3). In vivo footprinting of the $E_\beta$ (**A**), $E_\alpha$ (**B**), $A_\beta$ (**C**), and $A_\alpha$ (**D**) promoters on the coding and noncoding strands in M12.4.1 cells (M12) and BALB/3T3 (3T3) cells. (−) Naked DNA methylated in vitro; (+) DNA methylated in vivo. Plasmid DNAs containing promoter sequences were used to confirm the identity of the bands and to determine the regions contained in the Y, $X_1$, $X_2$, and S motifs (*14*), which are indicated by bars to the left of the lanes. Guanine residues protected from or hypersensitive to methylation are indicated by arrows to the right of the lanes. Adenine hypersensitivities are not indicated by arrows but represent bands seen in DNA methylated in vivo that are absent in DNA methylated in vitro. Identical results were seen in multiple experiments and with independently methylated samples. Preparation of methylated genomic DNA in vivo and in vitro and piperidine treatment were as described (*24*). Piperidine-treated genomic DNA (10 μg) was used in LMPCR reactions as described (*13*) with primers listed in (*25*).

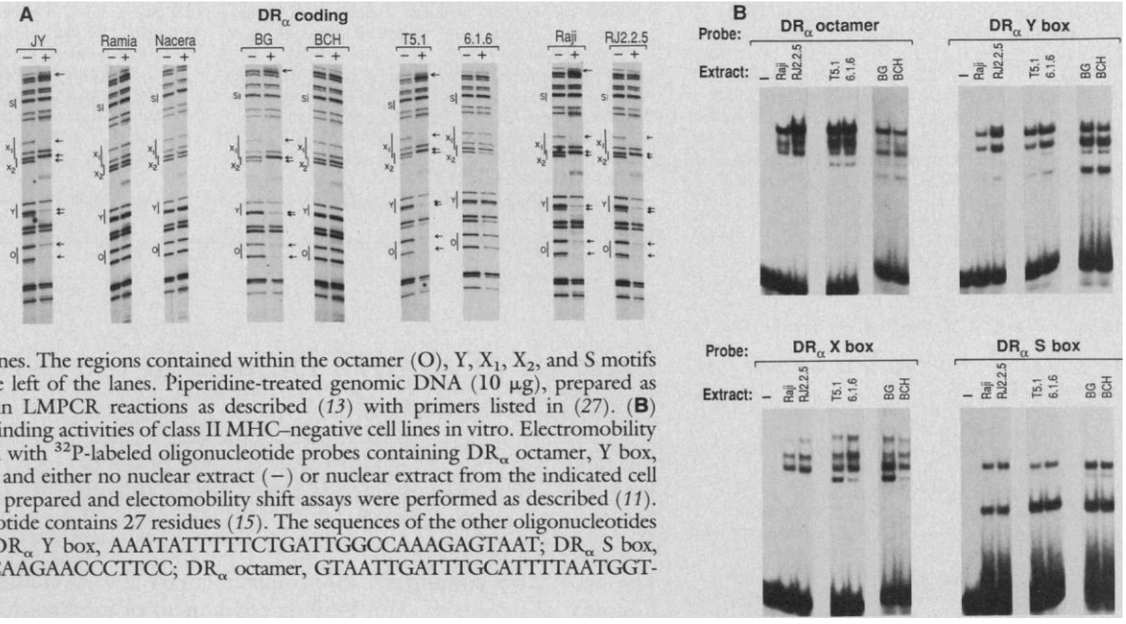


**Fig. 3.** (**A**) In vivo footprinting of the $DR_\alpha$ gene promoter coding strand in class II MHC–positive and class II MHC–negative cell lines. (−) Naked DNA methylated in vitro; (+) DNA methylated in vivo. DNA was from the cell lines indicated at the top of the lanes. Residues protected from, or hypersensitive to, methylation are indicated by arrows at the right of the lanes. The regions contained within the octamer (O), Y, $X_1$, $X_2$, and S motifs are indicated by bars at the left of the lanes. Piperidine-treated genomic DNA (10 μg), prepared as described (*24*), was used in LMPCR reactions as described (*13*) with primers listed in (*27*). (**B**) Examination of promoter binding activities of class II MHC–negative cell lines in vitro. Electromobility shift assays were performed with $^{32}P$-labeled oligonucleotide probes containing $DR_\alpha$ octamer, Y box, X box, or S box sequences, and either no nuclear extract (−) or nuclear extract from the indicated cell lines. Nuclear extracts were prepared and electomobility shift assays were performed as described (*11*). The $DR_\alpha$ X box oligonucleotide contains 27 residues (*15*). The sequences of the other oligonucleotides (5′ to 3′) are as follows: $DR_\alpha$ Y box, AAATATTTTTCTGATTGGCCAAAGAGTAAT; $DR_\alpha$ S box, TGTCCTGGACCCTTTGCAAGAACCCTTCC; $DR_\alpha$ octamer, GTAATTGATTTGCATTTTAATGGTCAG.

class II genes (Fig. 3A). The published $DR_\alpha$ sequence was derived from JY lymphoma cells (*18*), and therefore this cell line was examined as a positive control. The coding strand of $DR_\alpha$ in JY cells showed an equivalent footprint to that observed with the murine class II gene promoters, especially its homolog, $E_\alpha$. In addition, an octamer motif, which is unique to the $DR_\alpha$ promoter among all the class II genes, showed protection. Functional studies with transient transfections and in vitro transcription have demonstrated the importance of the octamer site for $DR_\alpha$ expression in B cells but not non–B cells (*19*). The S motif was not protected, as was the case with the murine genes. However, an adenine hypersensitivity was seen corresponding to position −150, which represents the 5′ end of the S region.

The class II MHC–positive cell lines BG, T5.1, and Raji had identical footprints to JY on $DR_\alpha$, in contrast to the class II MHC–negative cell lines Ramia, Nacera, BCH, and 6.1.6, which showed no protection at any of the identified sites. In addition, the adenine hypersensitivity at position −150 was absent in these lines (not visible in Fig. 3A for Nacera). The class II MHC–negative line RJ2.2.5 differed from the other mutant cell lines in that normal binding was apparent at the octamer, Y, $X_1$, and $X_2$ sites when compared to Raji. However, the adenine hypersensitivity at −150 was absent.

To ensure that these observations were not unique to the $DR_\alpha$ promoter but rather reflected the status of the class II gene promoters in these mutant cell lines in general, we examined a second human gene, $DR_\beta$ (*14*). Similar results were obtained with the $DR_\beta$ coding strand.

The defect in individuals with BLS and in experimentally derived class II MHC–negative cell lines has been attributed to trans-acting regulatory factors, but the primary lesion has not been determined for most of these lines. Nuclear extracts from Ramia and Nacera cells are missing a factor termed RFX, which binds to the $X_1$ site in $DR_\alpha$ in vitro, but the extracts do contain factors that can recognize the $DR_\alpha$ Y box and octamer site (*20*). To determine if any of the other mutant cell lines used in this study had any obvious defect in a promoter binding protein, we performed electromobility shift assays with nuclear extracts from the mutant cell lines RJ2.2.5, 6.1.6, and BCH, and their respective control cell lines Raji, T5.1, and BG, and with oligonucleotides encompassing either the octamer, Y, X, or S sites of $DR_\alpha$ (Fig. 3B). None of the bands present in the relevant class II MHC–positive control lines were absent in their class II MHC–negative counterparts. The amount of the fastest migrating protein-DNA complex with the X box oligonucleotide was reduced in 6.1.6 and BCH extracts as compared to their positive control lines; however, this band is completely absent from Raji extracts and therefore cannot be essential for class II gene expression. In addition, the second-fastest migrating complex in BCH was also diminished in intensity compared to BG. Further investigation of this observation will be necessary to determine if this reduction is relevant to the class II MHC–negative status of this cell line.

Our examination of a panel of class II MHC–negative regulatory mutants has demonstrated at least two distinct defects that affect gene expression by mechanisms other than direct promoter mutations. The RJ2.2.5 cell line had protein-promoter interactions both in vitro and in vivo, suggesting a defect in gene activation. A mutation in an activation domain of a transcription factor or in a coactivator protein that does not directly contact DNA could be responsible. In contrast, the Ramia, Nacera, BCH, and 6.1.6 cell lines showed no promoter interactions in vivo, despite the observation that such interactions occur in vitro. This finding suggests that the defects in these instances affect promoter accessibility and thus may involve chromatin structure. A mutation affecting a factor that interacts with a common locus activation region could prevent the organization of closed chromatin into an open domain (*21*). Alternatively, the defect may reside in a common transcription factor that serves not only to activate transcription but also to locally displace or reorganize nucleosomes (*22*). Elucidating the underlying mechanisms of the defects in these cell lines will provide a greater understanding both of BLS and of normal class II gene transcriptional regulation.

*Note added in proof*: We have since examined the DRα promoter in several other class II– mutant cell lines. BLS-1, SJO, and TF show the same phenotype as Ramia. BLS-2 and RM3 show the same phenotype as RJ2.2.5.

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25. The following oligonucleotides (5′ to 3′) were used for first strand synthesis (i), PCR (ii), and primer extension (iii), with the calculated melting temperature shown in parentheses. $E_\beta$ coding: (i) AGGGAACTCTGGGGAGCCACACCAT (64°C); (ii) GGAGAGGAGACACAGGAGTCAGAGG (64°C); (iii) GGAGACACAGGAGTCAGAGGGGAAGGC (68°C). $E_\beta$ noncoding: (i) TCTCACAAATATCCAGCTGCCTCTG (59°C); (ii) TGAGTGCTGGGATATGAGGCATGGG (62°C); (iii) GCTGGGATATGAGGCATGGGCCAGCAG (67°C). $E_\alpha$ coding: (i) GAACAGCAATGAAGAAAAATCTTAA (51°C); (ii) CTCCAATTGTGGCCATTTTCTTCTT (56°C); (iii) GCCATTTTCTTCTTGGGTGTTTGGTGGG (62°C). $E_\alpha$ noncoding: (i) ATTGGGACAGAAGATGTGTATTTTA (52°C); (ii) GAAATTTTTGTCCTGTTTGTCTACAGCC (56°C); (iii) primer (ii) was also used for primer extension. $A_\beta$ coding: (i) GATCTGCAGAGCCATCTCTAAGGCA (61°C); (ii) TAATGCCAGTCACCAGGACTCACAC (61°C); (iii) AGGACTCACACCTGCTGTGCCCCCTTC (67°C). $A_\beta$ noncoding: (i) TACTTCACGCACTTTTCTCTTTAAA (52°C); (ii) CATCCAGTGGGGCTCATGAAGAAAC (61°C); (iii) CCAGTGGGGCTCATGAAGAAACAGACC (64°C). $A_\alpha$ coding: (i) CGAGGACCCCCAGAATCAGAGCTCT (64°C); (ii) GGGAGGTCTCTGCAGCTGCTCTCCT (66°C); (iii) GGGAGGTCTCTGCAGCTGCTCTCCTGAG (68°C). $A_\alpha$ noncoding: (i) CAGAAGCACAAGAGAAAGGGTCTGA (59°C); (ii) CAGAGCCTGCAGCAGGTCCAGGGAG (67°C); (iii) GCCTGCAGCAGGTCCAGGGAGTTCCCC (70°C).
26. No band corresponding to the guanine at position −89 of the noncoding strand of $A_\beta$ was observed in LMPCR reactions with naked DNA as was expected from the published sequence (*23*). Subsequent sequencing of this region of genomic DNA revealed an adenine at this position, and thus the $A_\beta$ sequence in Fig. 2 reflects this finding. The guanine at position −70 of the coding strand of $A_\alpha$ was also not detected in LMPCR reactions with naked DNA. This was not due to an error in the published sequence, but rather may reflect some characteristic of the sequence that prevents completion of one of the steps in LMPCR before amplification of the fragments, and thus this fragment is "lost" from the final amplification products. Therefore, we cannot determine whether this residue is protected in in vivo methylated DNA.
27. The following oligonucleotides (5′ to 3′) were used for first strand synthesis, PCR, and primer extension, respectively, for the $DR_\alpha$ coding strand (melting temperature shown in parentheses): TCCACTTATGGCCATTTTCTTCTTG (56°C), TTGGGAGTCAGTAGAGCTCGGGAGT (62°C), and AGAGCTCGGGAGTGAGGCAGAACAGAC (65°C).
28. We thank C. Griscelli, B. Lisowska-Grospierre, M. Eibl, D. Pious, R. Accolla, C. Stiles, and S. Burakoff for cell lines; P. Mueller for advice on in vivo footprinting; M. Boothby and M. Grusby for helpful discussions; and P. Finn and M. Grusby for careful review of the manuscript. Supported by NIH grants GM36864 and A121163 and by the Leukemia Society of America.

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# Presence of an SH2 Domain in the Actin-Binding Protein Tensin

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**The molecular cloning of the complementary DNA coding for a 90-kilodalton fragment of tensin, an actin-binding component of focal contacts and other submembraneous cytoskeletal structures, is reported. The derived amino acid sequence revealed the presence of a Src homology 2 (SH2) domain. This domain is shared by a number of signal transduction proteins including nonreceptor tyrosine kinases such as Abl, Fps, Src, and Src family members, the transforming protein Crk, phospholipase C–γ1, PI-3 (phosphatidylinositol) kinase, and guanosine triphosphatase–activating protein (GAP). Like the SH2 domain found in Src, Crk, and Abl, the SH2 domain of tensin bound specifically to a number of phosphotyrosine-containing proteins from v-src–transformed cells. Tensin was also found to be phosphorylated on tyrosine residues. These findings suggest that by possessing both actin-binding and phosphotyrosine-binding activities and being itself a target for tyrosine kinases, tensin may link signal transduction pathways with the cytoskeleton.**

FOCAL CONTACTS (ALSO CALLED ADhesion plaques) are local, electron-dense regions of plasma membrane that contain vinculin, talin, integrin, fibronectin, actin, and other cytoskeletal components that mediate the attachment of many cultured cells to their substrate (*1*). Stress fibers composed of bundles of actin filaments are often anchored to the membrane in these regions. Growth factors, transforming oncogenes, and tumor promoters affect focal contacts and the organization of actin filaments (*2*). The composition, structure, and dynamics of focal contacts may therefore be influenced by the interaction between signal transduction pathways and the actin cytoskeleton.

Vinculin was initially thought to be the crucial linking factor that anchors actin filaments to the focal contacts. This assumption was based on evidence that certain vinculin preparations possessed actin filament capping and bundling activities (*3*). However, these activities were absent from highly purified vinculin preparations (*4*). Instead, they were found in a contaminating fraction of the cruder vinculin preparations (*5*). Antibodies to these contaminants recognized focal contacts in chicken embryo fibroblasts (CEF), Z-lines of skeletal muscle fibrils, and cultured embryonic heart cells (*5*). On protein immunoblots, the antibodies recognized two immunologically related species with apparent molecular sizes of 150,000 and 200,000, which are distinct from that of talin (*5*). Analysis of peptide maps showed that the 150-kD polypeptide is closely related to the 200-kD polypeptide (*5a*). The 150-kD polypeptide isolated from chicken gizzard, which was shown to interact with actin in vitro (*6*), was used for immunization of rabbits. The resulting polyclonal antibodies recognized focal contacts (*7, 8*) as well as both the 150-kD and 200-kD proteins. These two polypeptides are called tensin for their putative function in maintaining tension in the microfilaments at their point of anchorage.

The polyclonal antibodies against 150-kD tensin were used to screen a λgt11 cDNA library from CEF that contained short in-

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