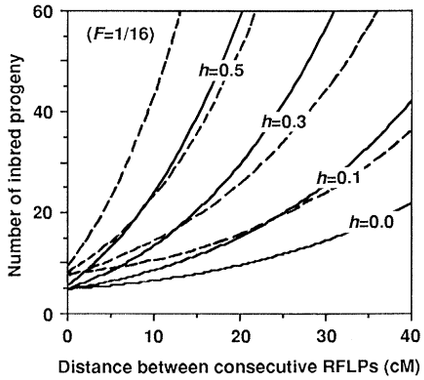

Fig. 3. Homozygosity mapping of a heterogeneous rare recessive trait by the method of simultaneous search (*19*). Number of first-cousin progeny required to identify a set of two loci at which disease alleles can occur, by means of RFLPs homozygous 50%, 30%, 10%, and 0% in the population, is shown in solid curves. Analogous result for a set of three loci is shown in dashed curves (unlabeled). Frequency of disease alleles at each of the loci is assumed to be equal.

sanguineous marriages are not uncommon and can be traced through records of the special dispensation that such marriages require from the Catholic Church (*16, 17*); Andhra Pradesh in India, where one-third of all Hindu marriages are between a niece and her maternal uncle (F = 1/8) (*18*); and many Middle Eastern populations (*15*). Note that the homozygosity rate h for an RFLP refers to the population from which the inbred is ascertained (*19*).

Homozygosity mapping clearly requires a reasonably complete genetic map of the human genome. An adequate map might consist of about 330 RFLPs evenly spaced every 10 cM, each homozygous at most 50% of the time in the population. Less dense maps would still be useful, while denser maps would allow linkage mapping with even fewer cases. Since more than 1000 RFLPs have already been discovered (in a human genome of about 3300 cM), such a map seems within reasonable prospect. Although the ideal map for homozygosity mapping is perhaps twice as dense as that for traditional family-based linkage studies (*20*), the smaller number of affected cases required for mapping should compensate for any increased effort in applying more markers. Moreover, one may first screen by means of a subset of the RFLPs and then confirm a suggestion of linkage by using a higher density of RFLPs in the region of interest.

The DNA of affected inbred children offers advantages not just for the initial detection of linkage, but also for the molecular cloning of the disease gene. While the surrounding region of homozygosity by descent is fairly large in any given child (median length ≈28 cM for the affected progeny of a first-cousin marriage), the search for the gene may be confined to the overlap of these regions (median length ≈28/n cM, if n affected first-cousin progeny are studied).

Finally, we have assumed above that the disease is homogeneous (is caused by mutations at a single locus), although it is hard to know this in advance. If linkage is not found under the assumption of homogeneity, however, one may adapt the strategy of simultaneous search, which we recently proposed elsewhere (*21*). Briefly, simultaneous search would consist of searching for a set of several loci with the property that at least one is homozygous by descent in most of the inbreds. Figure 3 shows the number of inbred individuals needed to map a heterogeneous trait by means of homozygosity mapping; for maximum efficiency, a higher resolution RFLP map would be preferred.

The study of inbred children will be more likely than traditional family studies to reveal rare trait–causing loci in the case of a heterogeneous trait, since loci will be represented in proportion to their allele frequency (q_1:q_2:···:q_k) among inbreds, but in proportion to the square of the allele frequency (q_1^2:q_2^2:···q_k^2) among the general population.

Homozygosity mapping may greatly extend the range of recessive diseases amenable to linkage mapping, because affected inbreds are not uncommon and relatively few are needed. Homozygosity mapping becomes practical only with the advent of a human genetic map. It exemplifies the sort of strategies for human genetics that will become available, we believe, as the human genome becomes more thoroughly explored (*20*).

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4. This point was apparently first noted by C. A. B. Smith [*J. R. Stat. Soc. B* **15**, 153 (1953)], who recognized the theoretical efficiency of studying inbred affected children, given a tightly linked, highly polymorphic marker. The paucity of such highly polymorphic markers and the inability at that time to construct complete linkage maps of less polymorphic markers led Smith to conclude that "the method is impractical." We thank an anonymous referee for calling our attention to this prescient insight.
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11. In practice, we would recommend preparing "immortal" lymphoblastoid cell lines from both the inbred, affected individual and his parents. Availability of parental DNA makes it easier correctly to infer the alleles at each locus, in order to detect homozygosity. Also, the expected LOD scores in the text increase slightly if parental DNA is included in the multipoint linkage analysis. If convenient, DNA from other relatives, such as grandparents, may also be included.
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Thrombospondin Promotes Cell-Substratum Adhesion

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The physiological role of the platelet-secreted protein thrombospondin (TSP) is poorly understood, although it has been postulated to be involved in platelet aggregation and cellular adhesion. In this report, TSP isolated from human platelets was found to promote, in vitro, the cell-substratum adhesion of a variety of cells, including platelets, melanoma cells, muscle cells, endothelial cells, fibroblasts, and epithelial cells. The adhesion-promoting activity of TSP was species independent, specific, and not due to contamination by fibronectin, vitronectin, laminin, or platelet factor 4. The cell surface receptor for TSP is protein in nature and appears distinct from that for fibronectin.

THE PLATELET-SECRETED PROTEIN thrombospondin (TSP) is thought to participate in platelet aggregation and cellular adhesion. TSP was first described by Baenziger *et al.* (*1*) and later shown to have a molecular weight of

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450,000 by sedimentation equilibrium analysis (*2*). SDS–gel electrophoresis revealed that TSP has three identical disulfide-linked polypeptide chains, each with an apparent molecular weight of 180,000 (*3, 4*). TSP is a major protein of the α-granule of platelets and accounts for approximately 25% of the total protein secreted by activated platelets (*5*). Other cells also produce TSP. Endothelial cells (*6–8*), fibroblasts (*9, 10*), and smooth muscle cells (*11, 12*) synthesize and release TSP and incorporate it into their extracellular matrix. The protein is present in human brain glial cells where its synthesis is stimulated by platelet-derived growth factor (*13*). In vivo, TSP has been identified in peritubular connective tissue, the basement membrane of human skin and lung, and the interstitial areas of human kidney and aorta (*14*).

Thrombospondin interacts with a number of macromolecules, including heparin (*15*), fibrinogen (*16–18*), fibronectin (*17, 19*), type V collagen (*12*), histidine-rich glycoprotein (*20*), plasminogen (*21*), and sulfated glycolipids (*22*). Distinct binding domains have been identified for fibrinogen (*23*), heparin (*24*), and type V collagen (*12*). In addition, calcium binds to TSP and affects its conformation, since treatment with EDTA profoundly alters the sedimentation coefficient, intrinsic viscosity, circular dichroism, susceptibility to proteolysis, and electron microscopic appearance of TSP (*2, 25*).

The evidence to support a role for TSP in platelet aggregation is sketchy. Leung (*26*) showed that antibodies to TSP blocked fibrinogen binding to platelets and partially blocked thrombin-induced platelet aggregation, and concluded that TSP potentiated platelet aggregation by cross-linking platelet-fibrinogen aggregates. Kao *et al.* (*27*) also showed that an antibody to TSP inhibited aggregation of platelets stimulated by low levels of thrombin. However, they found that purified, exogenously added TSP inhibited thrombin-induced platelet aggregation in a dose-dependent manner. TSP has been reported to bind to normal platelets (*28*), which have fibrinogen receptors, and to thrombasthenic platelets (*29*), which lack fibrinogen receptors; these findings suggest that the binding of TSP to platelets involves more than just its binding to fibrinogen. Consistent with these views are the observations of Gartner *et al.* (*30*), who showed that the TSP associated with both normal and thrombasthenic platelets agglutinated trypsin-treated erythrocytes.

Since TSP has been localized to the extracellular matrix (*6–12*), it seemed reasonable to speculate that TSP, like fibronectin, plays a role in cell-substratum adhesion. Encouraged by studies showing that TSP supports cell-substratum adhesion of malaria-infected erythrocytes (*31*) and that human melanoma cells adhere to TSP (*32*), we evaluated the ability of purified TSP to promote adhesion of platelets and various cell lines. We report here that TSP promotes the specific cell-substratum adhesion of a variety of cell types from several species, suggesting that TSP is a ubiquitous extracellular adhesion protein.

Like fibronectin, TSP promoted cell-sub-

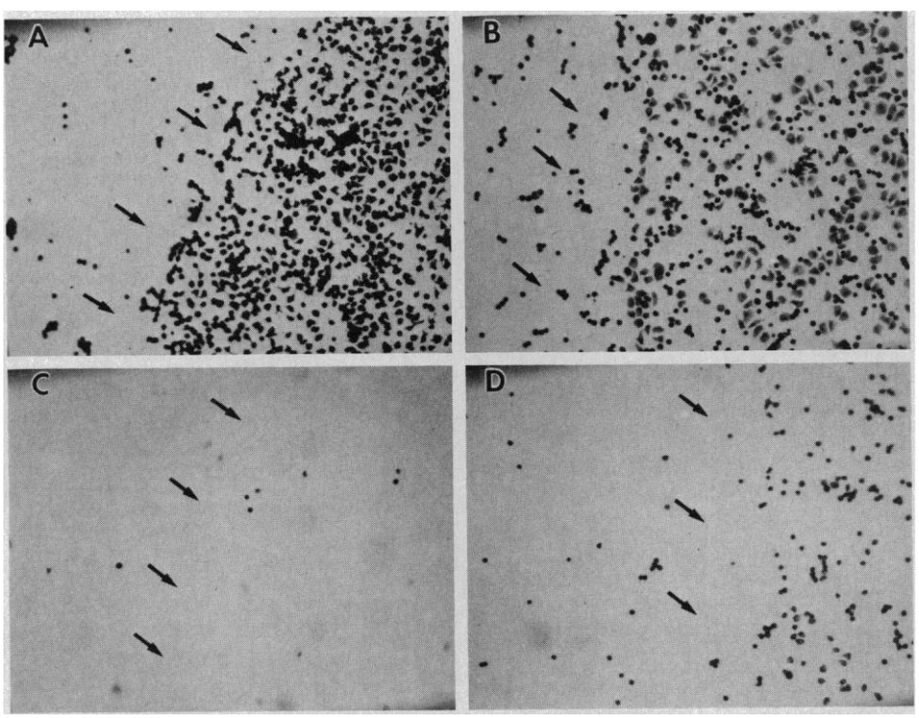

Fig. 1. Adhesion of B16-F10 mouse melanoma cells to TSP, fibronectin, BSA, and fibrinogen. Cell-substratum adhesion was performed as described (see Table 1). Each field (A to D) is representative of the cells adhering to a portion of the protein-coated area. The arrows indicate the boundary between the protein-coated area and the uncoated area. The slides were coated with (**A**) TSP, (**B**) fibronectin, (**C**) BSA, or (**D**) fibrinogen. (Cells were photographed at ×40.)

Table 1. Support of adhesion of various cells from different species by TSP. The adhesion assays on each cell type were done on different days, usually with different TSP preparations. Wells (1 cm^2) of eight-well glass tissue culture slides were treated for 30 minutes at room temperature with 5-μl drops (2 to 3 mm diameter) of solutions (40 μg/ml) of TSP, fibronectin (FN), fibrinogen (FBG), or BSA. Proteins were dissolved in 20 m*M* tris-bis-propane, *p*H 5.5, containing 2 m*M* $CaCl_2$ and 0.25*M* NaCl. Cells were harvested with EDTA and washed four times in serum-free Dulbecco's minimum essential medium (DMEM). Platelets were gel-filtered in Hepes-buffered Tyrode's solution, *p*H 7.0, containing 0.3% BSA and 2m*M* $CaCl_2$ as described (*16*). A 300-μl portion of resuspended cells containing 2.0×10^5 cells or a 300-μl portion of gel-filtered platelets containing 2.0×10^7 platelets was added to each well. The number of cells used saturated the surface of the well and provided a condition such that the number of cells was not a limiting factor in the assay. Cells were incubated for 30 to 60 minutes at 37°C and platelets for 30 minutes at room temperature. Nonadherent cells were removed by aspiration and the wells were washed with phosphate-buffered saline three times. Adherent cells were fixed with 2% glutaraldehyde for 10 minutes and stained with 2% Giemsa overnight. Cells were viewed at ×100 magnification and platelets at ×400 magnification, and representative fields in 1.0-mm^2 areas were counted.

Cell type	Species	Number of adherent cells per square millimeter*			
		TSP	FN	FBG	BSA
Melanoma (B16-F10)	Mouse	212	230	3	11
Fibroblasts (WI 38)	Human	247	500	33	2
Muscle (L6)	Rat	67	109	18	1
Epithelial ($LLC\text{-}PK_1$)	Pig	116	153	34	10
Endothelial (BFAE-39/6-3)	Cow	190	480	49	13
Platelets	Human	530	515	683	71

*In this and all other experiments variability in cell counts on different protein-coated 1-mm^2 areas within one well varied by less than 12%, and variability between duplicate wells within one experiment varied by less than 15%. All adhesion experiments were repeated two to ten times. Whereas the absolute number of cells in a 1-mm^2 field varied by as much as 55% from day to day, the variation was proportional, that is, more or fewer cells were observed across all treatment groups. The data presented in this table and in Tables 2 and 3 are from representative experiments.

stratum adhesion of mouse B16-F10 melanoma cells (Table 1 and Fig. 1). After 30 minutes, at a cell concentration of 5×10^4 cells per milliliter, more than 75% of the cells attached and were partially spread on either TSP or fibronectin. In contrast, the cells did not adhere to bovine serum albumin (BSA) and adhered only poorly to fibrinogen (Fig. 1 and Table 1), with the exception of the platelets (Table 1), which are known to bind fibrinogen (*33*). Adhesion to TSP was independent of cell type and species, since platelets, fibroblasts, skeletal muscle cells, and endothelial and epithelial cells from human, mouse, rat, cow, and pig all adhered to TSP (Table 1). We studied the B16-F10 cell line further to more fully establish the specificity of adhesion to TSP and to characterize the cell-surface TSP receptor.

The cell adhesion-promoting activity of our purified TSP was specific. For example, TSP adsorbed with antibody [immunoglobulin G (IgG)] to TSP did not promote cell adhesion, whereas TSP adsorbed with control antibody did (Fig. 2 and Table 2). The antibody to TSP was produced in a rabbit against the SDS-gel–purified 180,000 subunit of fibrinogen-Sepharose–purified TSP shown to be free of fibrinogen, β-thromboglobulin, fibronectin, and platelet factor 4 (*16*). In addition, this antibody has been shown to have no cross-reactivity with the proteins mentioned above (*5*). The specificity of TSP was further supported by the observation that B16-F10 cells assumed a different morphology on TSP than on fibronectin. As shown in Fig. 2, the cells are somewhat flattened on fibronectin, whereas on TSP they are more elongated. Additional experiments that show the specificity of TSP-mediated adhesion are summarized in Table 2. Treatment of cells with exogenous TSP before plating them on TSP also inhibited adhesion, which suggests that soluble TSP competes with immobilized TSP for cell binding sites.

To eliminate the possibility that the purified TSP used in these studies contained contaminating proteins, we routinely chromatographed fibrinogen-Sepharose–purified TSP preparations on Superose 6B (Pharmacia), which fractionates proteins in the molecular weight range of 5×10^3 to 4×10^6, with Pharmacia's Fast Protein Liquid Chromatography System. By gel filtration analysis a single peak was observed with a molecular weight of 902,000. When analyzed by SDS–gel electrophoresis, this peak contained a single polypeptide band of 180,000 molecular weight. Since the molecular weight of TSP as determined by sedimentation equilibrium analysis has been reported to be 450,000 (*2*), the higher molecular weight obtained by our gel filtration analysis might indicate asymmetry of TSP (*2*) or that TSP may exist as a dimer.

TSP preparations used in these studies were analyzed for the presence of fibronectin, vitronectin, laminin, and platelet factor 4, since all of these proteins have been reported to promote cell adhesion (*34–37*). No fibronectin was detected in our purified TSP by immunoblot analysis with a monoclonal antibody (Mallinckrodt) to fibronectin. Furthermore, no platelet factor 4 was detected by immunoblot analysis with a polyclonal antibody (Atlantic Antibodies) or by radioimmunoassay as previously described (*5, 16*). In addition, our purified TSP contained no detectable laminin, fibronectin, or vitronectin as measured by direct enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies against laminin (Calbiochem), fibronectin (Mallinckrodt), or vitronectin (Calbiochem). Positive controls in the ELISA and immunoblot assays included fibronectin (Sigma), vitronectin (Calbiochem), laminin (Collaborative Research), and platelet factor 4 (Sigma). Therefore, the homogeneity of our purified TSP as revealed by gel filtration and SDS–gel electrophoresis and the lack of significant contamination by laminin, fibronectin, vitronectin, and platelet factor 4 indicate that the cell adhesive activity of the purified TSP preparations is due to TSP.

We also characterized the cell-surface TSP

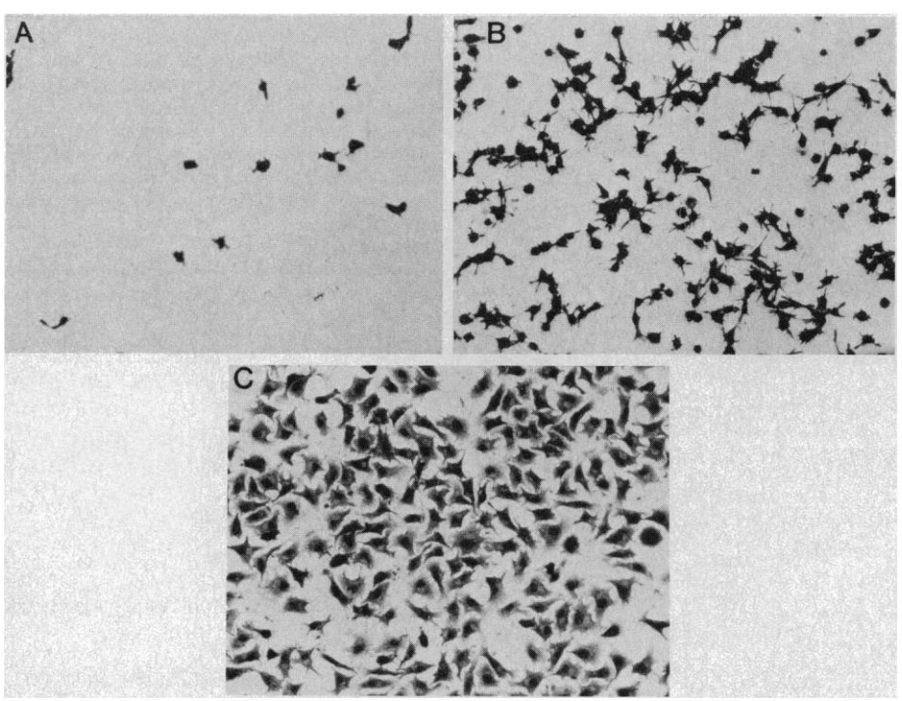

Fig. 2. Adhesion of B16-F10 mouse melanoma cells to TSP adsorbed with either IgG antiserum to TSP, or control serum IgG, and adhesion of cells to fibronectin. The cell-substratum adhesion assay with B16-F10 cells was conducted as described (see Table 1). Briefly, a portion (100 μl) of a solution of TSP (40 μg/ml, in 20 m*M* tris-bis-propane, *p*H 7.0, containing 2 m*M* $CaCl_2$) was passed through a 2 cm by 0.2 cm protein A Sepharose column that had been treated with 100 μl of either normal rabbit IgG (2 mg/ml) or a monospecific rabbit IgG antibody to gel-purified TSP (*5, 16*). (**A**) Cells adhering to the surface coated with TSP adsorbed with IgG antiserum to TSP. (**B**) Cells adhering to the surface of TSP adsorbed with control serum IgG. (**C**) Cells adhering to the surface coated with fibronectin (40 μg/ml). (Cells were photographed at ×100.)

Table 2. A monospecific antibody to TSP and soluble TSP inhibit adhesion of cells to immobilized TSP. B16-F10 mouse melanoma cells were harvested with EDTA and resuspended in serum-free DMEM, and adhesion assays were performed on TSP-coated slides as described in the legend to Table 1. In experiment 1, cells were treated with buffer or TSP (7 μg/ml) for 10 minutes at room temperature and then plated on TSP-coated slides. In experiment 2, TSP was adsorbed with control rabbit IgG or IgG antiserum to TSP prior to its being coated on the slide, as described in the legend to Fig. 2.

Treatment	Adherent cells per square millimeter	Percent of control
Experiment 1		
Buffer	1245	100
TSP	44	3.5
Experiment 2		
TSP adsorbed with rabbit IgG	424	100
TSP adsorbed with rabbit antiserum to human TSP IgG	2	0.5

Table 3. The effect of trypsin and antiserum treatment on adhesion of cells to TSP and fibronectin. In experiment 1, B16-F10 cells were treated with cycloheximide (20 μ*M*) for 60 minutes before being washed and then harvested with EDTA (1 m*M*) or EDTA (1 m*M*) plus trypsin (1 mg/ml). After 15 minutes at 37°C, soybean trypsin inhibitor (1 mg/ml) was added. Cells were resuspended in serum-free DMEM containing cycloheximide (20 μ*M*), and adhesion assays were performed on TSP- and fibronectin-coated slides (see Table 1). In experiment 2, cells harvested with EDTA in the absence of cycloheximide were treated with a 1:40 dilution of antiserum to GP140 or preimmune serum for 10 minutes at 37°C and plated on either TSP- or fibronectin-coated slides. The apparent potentiation of cell adhesion to TSP in the presence of antiserum to GP140 was a consistent finding and is not understood at this time.

Treatment of cells	Adherent cells per square millimeter	
	TSP	Fibronectin
Experiment 1		
EDTA	489	285
Trypsin/EDTA	3	111
Experiment 2		
Preimmune serum	505	923
Antiserum to GP140	1070	5

receptor. Cells were treated with trypsin in the presence of cycloheximide to prevent protein synthesis and then tested for their ability to adhere to TSP and fibronectin. A similar procedure was used by Tarone *et al.* (*38*) to characterize the cell fibronectin receptor. Trypsin treatment totally abolished cell adhesion to TSP, whereas it inhibited adhesion to fibronectin to a lesser extent (Table 3). These results suggest that the TSP receptor is protein and is more sensitive to proteolysis than the fibronectin receptor. We also treated cells with a polyclonal antiserum (antiserum to GP140) to cell surface adhesion–related glycoproteins of 140,000 molecular weight (*39*). This antiserum is a potent inhibitor of fibronectin-mediated cell adhesion. Cells treated with antiserum to GP140 adhered to TSP but not to fibronectin (Table 3). These data suggest that the molecular mechanism involved in TSP-mediated cell-substratum adhesion is distinct from that involved in fibronectin-mediated adhesion.

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Protein-Binding Sites in Ig Gene Enhancers Determine Transcriptional Activity and Inducibility

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Individual protein-binding sites within the mouse immunoglobulin heavy chain and kappa light chain gene enhancers were altered, making it possible to examine the functional role of the sites during transcription. The E motifs, which bind factors that are present in many if not all cells, mostly behave as transcriptional activating sites. The only known heavy chain enhancer site that binds a lymphocyte-specific factor, the "octamer" site, plays a critical role in transcription but only in a truncated form of the enhancer. In the full enhancer, no one site is crucial because of an apparent functional redundancy. The site in the kappa enhancer that binds a factor specific to mature B cells, κB, was crucial to the constitutive activity of the enhancer in B cells. This factor is also inducible in pre-B cells, and the site was necessary for inducibility of the kappa enhancer. Thus, the sites defined by protein binding are important for the functional activity of immunoglobulin enhancers, with the sites that bind proteins restricted in their cellular distribution playing the most important roles.

MATURATION OF B LYMPHOCYTES in adult bone marrow is a paradigm for differentiation of a cell lineage (*1*). At the pre-B cell stage, rearrangement and transcription of the immunoglobulin (Ig) heavy chain gene takes place (*2*). In more mature cells, light chain genes are rearranged and transcriptionally activated, and Ig tetramers are then expressed on the cell surface (*3*). Agents such as bacterial lipopolysaccharide, cycloheximide, and phorbol esters can induce kappa gene expression at earlier stages (*4*). In both the Ig heavy chain and kappa light chain genes, the large intron between the variable and constant regions contains a transcriptional enhancer element (*5*). These cis-acting sequences function independently of position and orientation and are virtually restricted to activity in cells of the B-lymphoid lineage although a portion of the heavy chain en-

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