tion? Is the antibody response to exogenous insulin, especially human insulin, different in patients depending on the presence or absence of antibodies before diagnosis? Since the immune response to exogenous insulin may vary depending on type of histocompatibility antigen (HLA), is there any association between the presence of insulin antibodies before insulin treatment and either of the HLA types associated with type 1 diabetes, Dr3 or Dr4?

In summary, we have found insulin antibodies in at least 18 percent of a group of untreated IDD's. The antibodies may be present in a larger percentage of diabetics, but with the current assay their values overlap with normal values. This measurement may prove superior to ICA's as a marker of B-cell damage, since cytoplasmic ICA is not specific for B cells (*23*), cell-surface ICA is present in a high percentage of nondiabetics (*24*), and both ICA measurements are difficult to perform and require subjective interpretation. The ease of the insulin-binding assay may make it useful for large-scale studies.

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Biotinated Probe Containing a Long-Terminal Repeat Hybridized to a Mouse Colon Tumor and Normal Tissue

Abstract. *The cloned complementary DNA pMCT-1, which contains an intracisternal A particle long-terminal repeat, is more highly expressed in a mouse colon tumor than in the normal mouse colon. In situ hybridization of biotin-substituted pMCT-1 to fixed frozen sections shows that expression of pMCT-1 is seen throughout the tumor and is highly heterogeneous on a cellular basis, while expression is undetectable in any cell in the normal colonic mucosa.*

The complementary DNA clone pMCT-1 (*1, 2*) contains a mouse highly repetitive intracisternal A particle long-terminal repeat (*3*). This clone hybridizes to a population of transcripts that are heterogeneous in size and that show 50-fold higher expression in the dimethylhydrazine-induced transplantable mouse colon tumor from which it was derived (*4*) than in the normal mouse colonic mucosa (*2*). Expression is also high in several leukemia cell lines (*2, 5*). In the colon tumor RNA molecules that hybridize to pMCT-1 comprise approximately 0.8 percent of the polyadenylated RNA population.

As in all experiments that detect changes in gene expression in complex tissues, the question arises as to whether the differences seen between the colon tumor and normal colon represent alterations in the distribution of cell types. In situ hybridization of biotin-substituted pMCT-1 to frozen sections of tumor and normal tissues demonstrates that not only is expression undetectable in any cell in the normal colonic mucosa, but that expression is highly heterogeneous throughout the transplantable tumor. The results presented here illustrate the value of complementing investigations on gene expression with methods in which the architecture of the tissue and spatial relations between cells are preserved.

Frozen sections of freshly dissected colon tumor 36 grown in male BALB/c mice (*4*), normal mouse colon, and liver were hybridized to biotin-substituted pMCT-1 and pBR322, as described in the legend to Fig. 1. The photographs in Fig. 1 show sections fixed for 5 minutes in cold Carnoy's B fixative (60 percent ethanol, 30 percent chloroform, and 10 percent acetic acid) and briefly incubated at room temperature with autodigested Pronase (Calbiochem). In our experiments the use of 4 percent paraformaldehyde (*6, 7*) or ethanol and acetic acid (3:1 by volume) (*8*) as fixatives did not preserve morphology as well in frozen sections, but recent results show that 4 percent paraformaldehyde for 30 minutes at room temperature is the fixative of choice for Friend erythroleukemia cells in culture. Brief Pronase digestion is necessary to obtain optimum hybridization and hence signal, but without careful monitoring during this step, overdigestion and destruction of tissue can result. Detection of hybridization was primarily by indirect immunofluorescence.

The morphology of the cells and crypts of the normal mouse colon was well preserved (Fig. 1A). In 11 experiments with 37 sections from 7 normal mouse colons, no signal was ever detected with biotin-substituted pMCT-1. These results are not shown since they yield completely black photographs due to the necessity of filtering out all but the emitted light to record the signal. In contrast, hybridization of biotin-substituted pMCT-1 to the colon tumor always resulted in fluorescence throughout the tumor (Fig. 1C). A similar signal was seen in 13 sections from 8 different tumors. In addition, however, localized bright areas were usually seen, and in

over half the sections (7 of 13 sections from 8 tumors) there were also intensely staining doublets (arrows in Fig. 1C). As a control, biotin-substituted pBR322 [the vector into which the complementary DNA molecule was cloned (*1*)] never produced a signal in any tissue.

The heterogeneity of expression of pMCT-1 in the tumor is noteworthy. Figure 1B shows a photograph of the same section as in Fig. 1C made with transmitted light. The tumor is morphologically homogeneous, as determined from sections fixed with Formalin and stained with hematoxylin and eosin (*9*). Therefore, in situ hybridization reveals a heterogeneity of expression of transcripts that hybridize to pMCT-1 which is not reflected in the histology of the tissue. One possible explanation for this heterogeneity stems from our work on the expression of pMCT-1 in Friend erythroleukemia cells. Expression is highest in Friend cells arrested in early G_1 (*5*). The doublets seen in the mouse colon tumor (Fig. 1C) may, therefore, represent cells that have just divided, and hence each daughter cell is transiently in early G_1. Note also that the fluorescence of the doublets decreases as they are separated by greater distance, possibly as they proceed through the cell cycle.

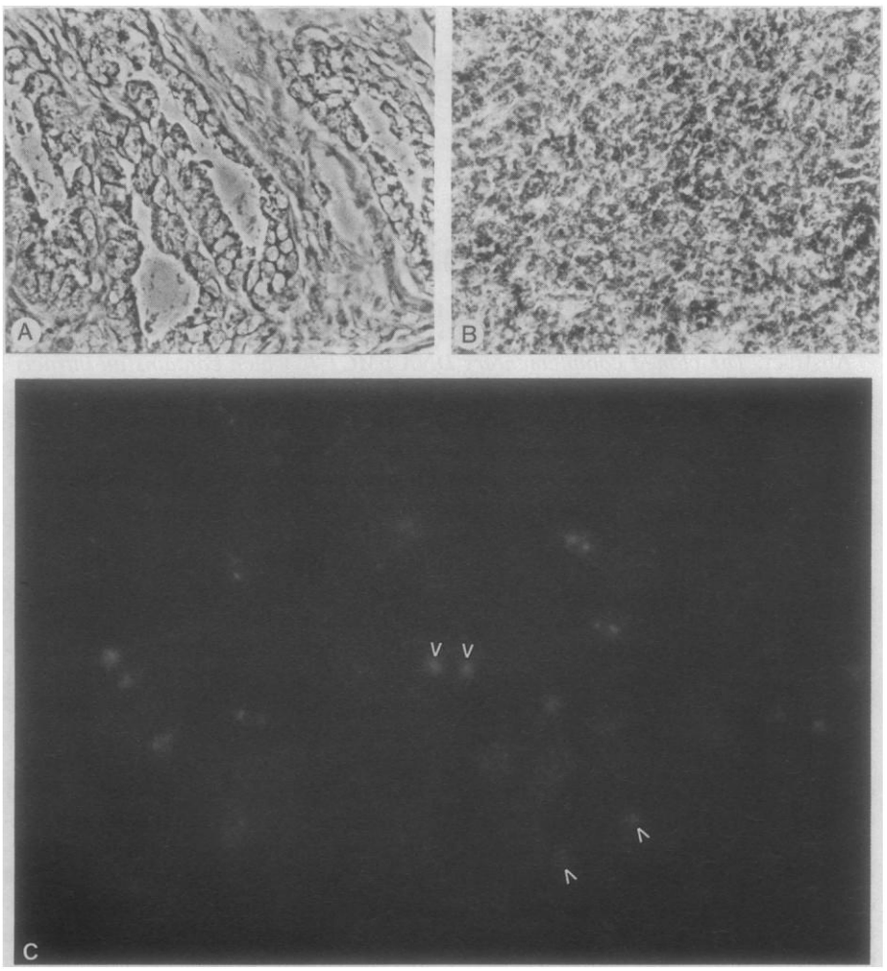

Fig. 1. Photomicrographs of frozen sections of mouse tissues. The animals were killed by cervical dislocation and dissected tissues were immediately embedded by freezing in O.C.T. compound (Lab Tek Products). Sections measuring 8 μm were cut on a cryostat at −19°C, mounted on albumin-subbed slides (*7, 15*), fixed for 5 minutes at −20°C in Carnoy's B fixative, dried at the same temperature, and stored at −70°C. The sections were rehydrated for 20 minutes in 0.05*M* tris (*p*H 7.6) and then digested with Pronase in 0.05*M* tris (*p*H 7.6) and 5 m*M* EDTA for 5 minutes at room temperature. The digestion was stopped by washing the sections twice for 3 minutes in 0.005*M* tris (*p*H 7.6) and glycine (2 mg/ml). After dehydration through an ethanol series, each section was hybridized with a biotinated probe (3 ng/μl) in 15 μl of a solution containing 50 percent formamide, 10 percent freshly prepared dextran sulfate, a fivefold concentration of SSC (SSC contains 0.15*M* sodium chloride and 0.015*M* sodium citrate; *p*H 7.0), and denatured salmon sperm DNA (100 μg/ml). Probes were biotinated by nick translation with biotin-substituted deoxyuridine 5′-triphosphate (Enzo Bio-Probe Detection System, Enzo Biochemicals). Hybridization was carried out at 37°C under sealed cover slips for 18 hours. The sections were then washed in a twofold concentration of SSC for 5 minutes at 32°C, washed again in 0.1 percent Triton X-100 in phosphate-buffered saline (PBS) for 5 minutes at room temperature, and finally rinsed in PBS. At this point the slides were not permitted to dry. For detection the sections were incubated with a 1:100 dilution of goat antiserum to immunoglobulin G (IgG)–linked biotin (Enzo Bio-Probe) in PBS plus bovine serum albumin (2 mg/ml) for 60 minutes at 37°C in a humid chamber and washed three times for 5 minutes in PBS. They were then incubated with a 1:100 dilution of fluorescein isothiocyanate–conjugated rabbit antiserum to goat IgG (Enzo Bio-Probe) and washed as above. The sections were counterstained for 30 seconds in 5 mg of Evans blue per 100 ml of PBS, washed for 10 minutes in PBS, and stored under sealed cover slips in 50 percent glycerol and 50 percent PBS. They were viewed with a Zeiss fluorescence microscope at 455 nm or with transmitted light and photographed with Ektachrome 400 daylight film. (A) Normal mouse colon viewed with transmitted light. (B) Mouse colon tumor 36 hybridized with pMCT-1 and viewed with transmitted light. The tissue appears homogeneous. (C) Fluorescence microscopy of the same slide as in (B). Note the overall fluorescence, with bright areas and intensely fluorescing doublets (examples are depicted with arrows). Final magnification, ×640.

We also used an avidin–horseradish peroxidase detection technique with sections hybridized to biotin-substituted pMCT-1 (Vectastain ABC kit, Vector Laboratories). In this case the signal was localized to discrete, fine, dark lines in the cytoplasm of the cells, often circling the margin of the nucleus. This pattern may reflect the localization of A particles in the cisternae of the endoplasmic reticulum (*10*).

The results show that transcripts which hybridize to pMCT-1 are not expressed at a detectable level in any of the many cell types found in sections of normal mouse colon. Biochemical results (*2*) demonstrate that the level of expression is at least 50-fold higher in the colon tumor, and the work described here shows that such transcripts are present throughout the tumor but in a highly distinct heterogeneous fashion. The biochemical findings also reveal that hybridization is slightly higher in the liver than in the normal colon, and this is confirmed by in situ hybridization, since a very faint signal is seen throughout the liver.

For two major reasons, in situ hybridization has the potential to complement research on gene expression in transformation and carcinogenesis. First, such hybridization enables one to visualize early changes in small numbers of cells or in single cells that might otherwise be diluted in preparing tissue homogenates. This is important because tumorigenesis is a multistep process and occurs over a long period (*11*). Second, although they may appear histologically homogeneous, tumors are composed of heterogeneous populations of cells with various phenotypes (*12*). These differences in charac-

teristics (such as malignant potential and drug resistance) may depend on differences in gene expression that can be revealed by in situ hybridization with appropriate probes. The method of using biotin-substituted probes (*6, 7, 13, 14*) is particularly elegant since, once synthesized, such probes are stable and detection does not require lengthy exposure times. The usefulness of such probes will increase as methods are developed for improved sensitivity of detection and more accurate quantitation of the signal.

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Correlation of Glucocorticoid Receptor Binding Sites on MMTV Proviral DNA with Hormone Inducible Transcription

Abstract. *Steroid hormones, when complexed to their receptors, recognize and bind specific DNA sequences and subsequently induce increased levels of transcription. The mechanisms of steroid hormone action were analyzed by constructing chimeric DNA molecules from portions of mouse mammary tumor virus envelope and long terminal repeat (LTR) regions ligated to the thymidine kinase (tk) gene of herpes simplex virus. This construction allowed the tk gene to be expressed in a hormone-responsive fashion upon transfection into Ltk$^-$ cells. Comparison of transcription data with in vitro binding data showed that hormone-responsive transcription can be directly correlated to the presence of steroid hormone receptor binding sites on the DNA. There are at least two such receptor binding sites in the LTR region, one between −202 and −137 and another between −137 and −50 base pairs from the RNA cap site, as well as a site near the 5′ end of the envelope region. These results strengthen the hypothesis that steroid-receptor complexes regulate genes primarily by binding to DNA sites near the promoter region and thereby modulate transcription.*

Steroid hormones bind specific cytoplasmic receptor molecules which then undergo an activation process and accumulate in the cell nucleus. The activated steroid-receptor complex, which is thought to act as a modulator of gene expression, is capable of recognizing specific DNA sequences near the promoter region of genes that are under steroid hormone control (*1–6*). This suggests that such specific binding sites on the DNA have a primary role in the modulation of gene expression by the steroid-receptor complex. The mechanism of gene regulation by steroid hormones in eukaryotic cells might therefore be similar to prokaryotic gene regulation wherein regulatory proteins bind to a specific DNA sequence in the promoter region of genes under their control, thereby changing the rate of transcription.

Mouse mammary tumor virus (MMTV) serves as a convenient system in which to analyze the site or sites necessary for steroid hormone regulation. This system has been used to study both in vitro binding (*1, 4–6*), involving DNA restriction enzyme fragments and the glucocorticoid-receptor complex, and in vivo transcription, in which cell cultures are transfected with the desired DNA fragments (*7–9*).

In the experiments described here we attempted to correlate previously obtained transcription data (*9*) with DNA binding data to define the sequence requirements for gene expression induced by the glucocorticoid receptor. For this purpose we analyzed a set of chimeric DNA molecules in vitro for the presence of glucocorticoid receptor binding sites. The chimeric DNA molecules contain proviral MMTV sequences, including part of the envelope (env) gene and long terminal repeat (LTR), as well as the thymidine kinase (tk) gene of herpes simplex virus (HSV) (see Table 1). These chimeric genes, which lacked increasing length of sequences from the LTR, had previously been introduced into cultured L cells, and analysis of their transcripts had enabled investigators to define the LTR regions necessary for hormone-responsive transcription. Hormone-sensitive transcripts initiating correctly in the tk gene were also observed (Table 1) (*9*).

In Table 1 we show the DNA sequences present in the chimeric molecules and point out the DNA fragments that were assayed. Eco RI restriction fragments from the six different plasmids, containing various amounts of DNA from the MMTV env and LTR regions and 222 base pairs (bp) of mouse genomic DNA, were purified in microgram quantities and used in the DNA-cellulose competition assay (*4*). In this assay, ^{3}H-labeled triamcinolone acetonide (^{3}H-TA)–receptor complex is mixed with a DNA-cellulose suspension, where the cellulose-bound DNA is nonspecific (calf thymus) DNA. The amount of hormone-receptor complex bound to DNA-cellulose can be easily determined by pelleting the DNA-cellulose and counting the radioactivity associated with it. If free DNA is mixed with the DNA cellulose, the free DNA competes with the DNA cellulose for labeled steroid-receptor complex. DNA fragments containing specific binding sites for the glucocorticoid-receptor complex compete more strongly than nonspecific DNA fragments (*4*). Typical competition curves from the DNA-cellulose binding assay are shown in Fig. 1. Eco RI fragments from plasmids −236, −202, and −137 (Fig. 1, A and B) compete approximately five times better than nonspecific DNA [in this case, a 750-bp (Fig. 1D)