Identification by Cell Fusion of Gene Sequences That Interact with Positive Trans-Acting Factors

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Cell fusion experiments have implicated positive or negative regulatory factors in the cell type–specific expression of specialized endogenous genes. The inability to readily manipulate such genes has prevented characterization of the cis-acting DNA sequences that interact with these factors. A transfection-fusion technique, which combined stable gene transfer and formation of transient heterokaryons, was used to study this class of factors and their DNA binding sites. Messenger RNA directed by a quiescent, rat prolactin promoter region stably transferred into mouse fibroblasts was detected only after fusion to rat pituitary cells, implying that pituitary cells contain a positive cell type–specific factor or factors. Nuclear run-on assays showed that fusion activation is transcriptional. Fusion did not activate either a stably transferred rat growth hormone gene promoter or expression of the endogenous silent fibroblast prolactin or growth hormone genes. Analysis by 5′-deletion mutation identified a 30–base pair DNA sequence required for cell fusion activation of the rat prolactin promoter region. Comparison with previous results from direct cellular transfer of this region implies that transfection-fusion identifies novel regulatory DNA sequences.

EARLY INVESTIGATIONS WITH STABLE hybrid cell lines implicated both repression and activation mechanisms in the cell type–specific expression of specialized genes (*1*). More recent investigations that used the formation of heterokaryons have yielded evidence for the involvement of positive trans-acting factors in regulation of cell type–specific expression of muscle-specific (*2*) and globin (*3*) genes. However, further characterization of such factors and their target DNA sequences has been hampered by the absence of general techniques for directed mutagenesis of endogenous genes.

Expression of the genes for prolactin and growth hormone is limited to a specific set of pituitary gland cells (*4*). Previous experiments in which cloned genomic constructs were transferred into various cell types have shown that the 5′-flanking regions of the bovine prolactin gene (*5*) and the rat genes for prolactin and growth hormone (*6*) contain information for pituitary cell–specific expression. It was reported recently that fibroblasts contain a repressor that prevents expression of a transferred rat growth hormone gene region (*7*). For the prolactin gene, however, the question of whether positive or negative factors mediate pituitary cell–specific expression has not yet been addressed.

Cell fusion of heterologous cell lines produces transient heterokaryons in which the nuclei of the parental cell types remain separate (*3*) but are exposed to diffusible regulatory factors from the cytoplasm and nucleus of each cell type. Thus, if transient fusion between pituitary cells and fibroblasts activated fibroblast expression of a previously silent prolactin gene promoter construct, this would imply that pituitary cells contain one or more positive, diffusible cell type–specific regulatory factors for this gene. Mutation in vitro of this region could then identify the DNA sequence or sequences that interact with such a factor or factors. We report here the use of a transfection-fusion assay to identify such a DNA sequence, between 175 and 204 bp upstream of the rat prolactin gene cap site.

Parental plasmids used contain the promoter region of the rat genes for either prolactin (pPRL-CAT) or growth hormone (pGH-CAT), upstream of a chloramphenicol acetyltransferase (CAT) construct (Fig. 1). These constructs or derivatives were stably transfected into mouse fibroblast C127 cells, and pools were formed to average out the effects of insertion site in individual clones (*8*). For each construct examined, a polyclonal culture was then fused (*9*) to rat pituitary GH_3 cells, which express their endogenous prolactin and growth hormone genes (*10*). No CAT messenger RNA (mRNA) product (*11*) of a pPRL-CAT derivative (5′Δ-958) was detectable in either homogeneous or heterogeneous unfused cultures of GH_3 cells or transfected C127 cells (Fig. 2A). In contrast, fusion of a heterogeneous cell culture induced the accumulation to about four copies per cell of a correctly initiated CAT gene transcript. Analysis of other aliquots of the same RNA samples showed that fusion did not induce detectable expression by the endogenous mouse fibroblast genes of the mRNA for either prolactin (Fig. 2B) or growth hormone (Fig. 2C) (*12*). These results imply that transient fusion to pituitary cells can activate an exogenous prolactin promoter in fibroblasts. However, the genes for endogenous fibroblast prolactin and growth hormone, which were silenced during development, apparently cannot be activated by this procedure.

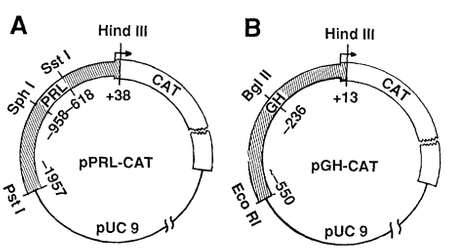

Fig. 1. Structure of parental recombinant plasmids pPRL-CAT and pGH-CAT. Plasmid pPRL-CAT contains the DNA sequence of the rat prolactin gene (*27*) between positions (relative to the prolactin cap site) −1957 and +38 (the latter formerly an Ava II site) linked to the Hind III–Bam HI fragment of the CAT gene (*16*), cloned into pUC 9 (*19*). Plasmid pGH-CAT, similarly constructed, contains the DNA sequence of the rat growth hormone gene (*24*) between positions ~−550 and +13 (the latter formerly an Xho I site) (*19*).

Results with a nuclear run-on assay (*13, 14*) showed that cell fusion activates transcription (Fig. 3). Fusion yielded a strong increase in transcription of CAT mRNA from a pPRL-CAT derivative, 5′Δ-618. Treatment with α-amanitin and heparin showed that transcription of this construct in fused cells was, respectively, directed by RNA polymerase II and initiated in the intact cells. Fusion did not increase transcription of the cotransferred construct pRSV-NEO, which showed that the results with 5′Δ-618 are promoter-specific. Finally, as would be anticipated, cell fusion did not affect the rate of transcription of the endogenous rat pituitary cell genes for either prolactin or growth hormone.

The transfection-fusion assay as used here is not strictly quantitative, since there is no adequate internal control for the extent of fusion of heterologous cells. However, as illustrated in Fig. 4, we used constructs containing 5′ deletions (*15*) and the sensitive CAT enzymatic assay (*16*) to investigate the prolactin promoter DNA sequence or sequences involved in activation by cell fusion. In three of four similar experiments, CAT expression was observed to decrease and increase, respectively, when regions up-

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stream of the prolactin gene between positions −395 to −317 and −316 to −232 were removed. This result, together with the significant expression by deletion mutant 5′Δ-316 in unfused fibroblasts (legend to Fig. 4), suggests that positions −395 to −232 contain both positive and negative regulatory elements, none of which are absolutely required for fusion activation (*17*). In all of the experiments of this type, deletion mutant 5′Δ-187 consistently exhibited one-tenth (or less) the CAT activity of mutant 5′Δ-204. Furthermore, 5′-deletion mutants containing 174 bp or less of 5′-flanking prolactin gene sequences consistently failed to be activated by cell fusion. The last observations imply that adjacent prolactin gene DNA sequences that are, respectively, partially and absolutely required for activation by cell fusion lie between positions −204 and −188 (sequence I) and −187 to −175 (sequence II) (or possibly spanning position −175). The region required for complete activation by transient cell fusion (positions −204 to −175) corresponds to a pituitary-specific chromosomal site of nuclease hypersensitivity at −215 to −180 (*18*), implying that the latter is created by the binding of pituitary cell–specific positive trans-acting factors to a complex regulatory region of the prolactin gene promoter.

Rat growth hormone gene construct pGH-CAT (Fig. 1) is expressed strongly in stably transfected GH_3 cells and at a 150-fold lower level in C127 cells (*19*). However, fusion of transfected C127 cells to GH_3 cells did not activate expression of either pGH-CAT or its 5′-deletion mutant 5′Δ-236 (Fig. 4). This result is consistent with the recent report that fibroblasts contain a repressor of the rat growth hormone gene (*7*). Thus, although the prolactin and growth hormone genes are evolutionarily related (*20*) and are each expressed specifically in pituitary cells, different mechanisms must mediate cell type–specific expression of the two genes.

The rat prolactin regulatory DNA sequences defined in the present studies are: sequence I, (5′ −204)GCTGTAATTAATCAAAA(3′ −188); sequence II, (5′ −187)TCCTTCCTTTCTG(3′ −175). Prolactin-related genes contain similar sequences, which may serve analogous functions. The last 13 nucleotides of sequence I, of which 12 are A or T, are perfectly conserved at similar locations in the human [position −215 (*21*)] and bovine [−213 (*22*)] prolactin genes. These 13 nucleotides also partially (10 out of 13) match sequences at an almost identical position in the rat growth hormone–releasing factor gene [−217 (*23*)], and at +153 of the human prolactin gene and +924 of the rat growth hormone gene (*24*). The first 12 nucleotides of sequence II, all pyrimidines, are partially (9 out of 12) homologous to sequences at similar locations in the bovine prolactin gene (−196 and −220), and at −862 in the human prolactin gene. These 12 nucleotides are partially (≥9 out of 12) homologous to rat growth-hormone gene sequences at +327 and +1420, human growth-hormone gene sequences at +1195, +1219, and +1304 (*25*), and sequences in the human chorionic somatomammatropin-1 gene [+1273 and +1383 (*26*)].

The 5′-deletion mapping by the present transfection-fusion assay (Fig. 4) apparently detects pituitary cell–specific regulatory DNA sequences different from those detected by a direct gene transfer assay (*6*). Prolactin 5′-flanking sequences I and II defined by cell fusion were not detected after direct gene transfer. Conversely, removal of the DNA region (−1530 to −1769) of this gene required for expression by direct gene transfer did not decrease (and actually increased) expression after cell fusion; this increase was possibly caused by either the presence in fibroblasts of an inhibitory fac-

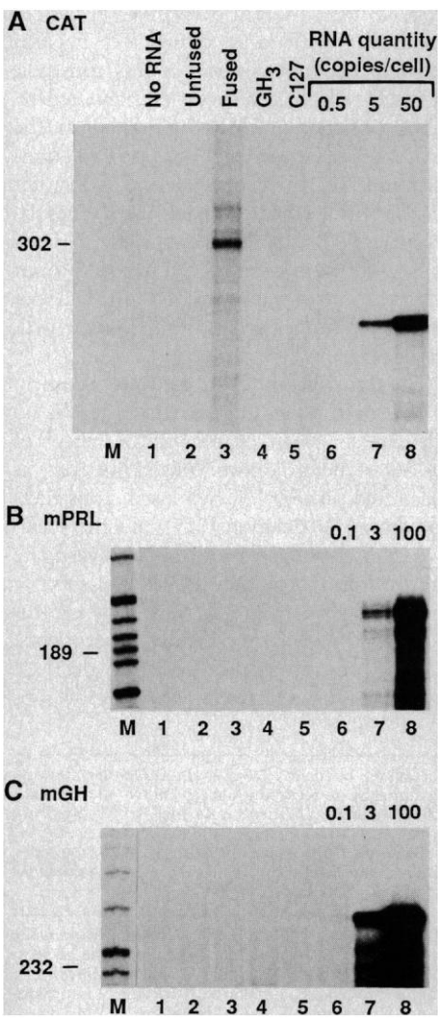

Fig. 2. Induction by cell fusion of accumulation of correctly initiated prolactin-CAT transcripts. A polyclonal culture of C127 cells stably transfected with pPRL-CAT deletion mutant 5′Δ-958 was cocultivated with GH_3 cells. Two days after fusion of some heterocultures by incubation with PEG (*9*), the indicated mRNAs were analyzed by ribonuclease protection analysis (*11*). Results similar to those shown were obtained in two other experiments. (**A**) Analysis of the CAT mRNA product of 5′Δ-958. A correctly initiated transcript should yield a protected probe of 302 nucleotides (*11*). Exposure to x-ray film was at −70°C with intensifying screen for 12 hours. Lane M, DNA size markers; lane 1, no input RNA; lanes 2 and 3, 10 μg of poly(A)$^+$ RNA from a heteroculture incubated in the absence (unfused) or presence (fused), respectively, of PEG; lanes 4 and 5, 10 μg of poly(A)$^+$ RNA from unfused GH_3 cells and C127 cells, respectively; lanes 6 to 8, internal standards for transcript quantitation. Exposure to film for 7 days still yielded no detectable protected probe in lane 2, implying that CAT mRNA was present in the unfused heteroculture at <0.1 copy per cell. (**B** and **C**) Analysis of mouse mRNAs for, respectively, prolactin and growth hormone, by means of a 24-hour film exposure. Expected sizes of protected probes are shown.

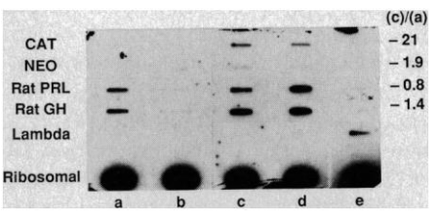

Fig. 3. Cell fusion activates transcription directed by an exogenous prolactin promoter. A polyclonal culture of C127 cells stably transfected with pPRL-CAT deletion mutant 5′Δ-618 was cocultivated with GH_3 cells, then incubated in the presence or absence of PEG, as described in Fig. 2. Three days later, ~10^7 nuclei from each culture were assayed by nuclear run-on transcription (*14*) for RNA polymerase II density on the indicated genes. The immobilized, gel-purified DNAs were as follows: CAT, the pSV2CAT (*16*) Hind III–Mbo I fragment; NEO, the pSV2NEO (*28*) Hind III–Sma I fragment; rat PRL, a Pst I–Pst I rat prolactin gene fragment from plasmid G (*29*); rat GH, rat growth hormone cDNA plasmid pBR322-GH1 (*30*) (not gel-purified); lambda, bacteriophage λ genomic DNA (not gel-purified); ribosomal, the rat 18*S* ribosomal RNA genomic plasmid pXC-1 (*31*) Hind III–Hind III fragment. Lanes a and b, nuclei from a heteroculture incubated without PEG were incubated in the absence or presence, respectively, of α-amanitin (1 μg/ml); lanes c, d, and e, nuclei from a heteroculture incubated with PEG were incubated in the presence of, respectively, no additions, heparin (1 mg/ml), or α-amanitin (1 μg/ml). (c)/(a), fold change in RNA polymerase II density, calculated by dividing (gene signal minus λ signal) from filter c by this quantity from filter a. A total of ~5×10^7 count/min was incorporated into RNA in nuclei analyzed in lanes c and a. For ribosomal, density was not determined. Results similar to those shown were obtained in two other experiments, in which no consistent difference in total counts per minute incorporated by nuclei from fused or unfused cells was observed.

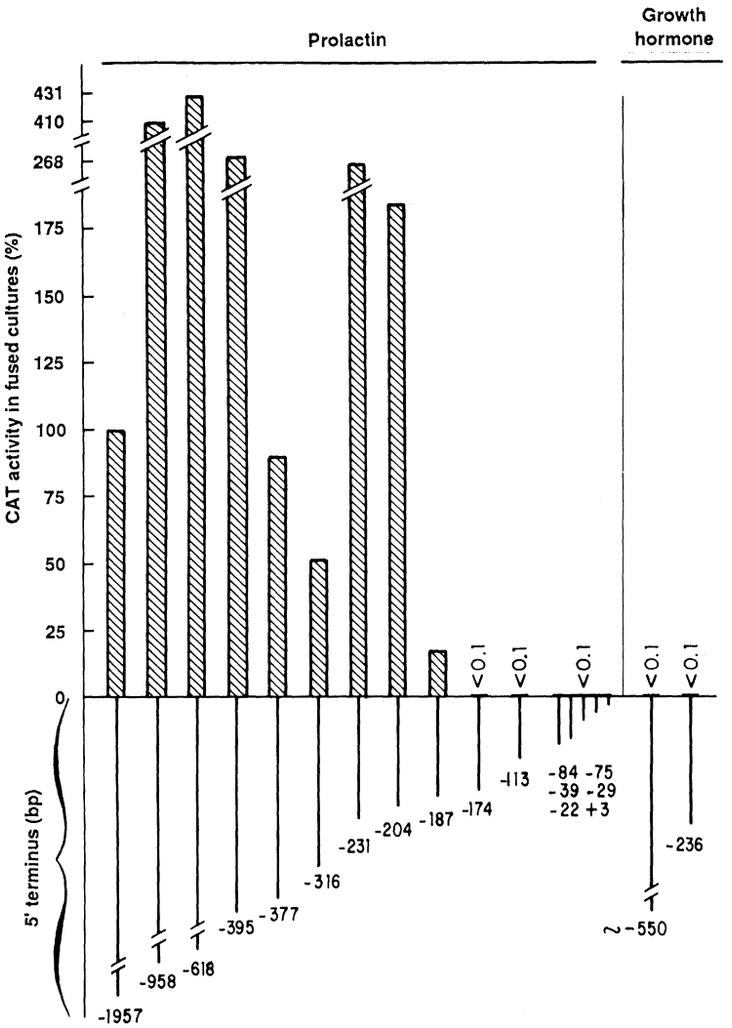

Fig. 4. Analysis by 5′-deletion mutation of cell fusion activation of the prolactin and growth-hormone promoter regions. Polyclonal cultures of C127 cells stably transfected with pPRL-CAT, pGH-CAT, or their indicated 5′-deletion mutants (*15*) were cocultivated with GH_3 cells, then briefly incubated in the absence or presence of PEG, as described in Fig. 2. After a 2- to 3-day incubation, CAT enzymatic activity was assayed (*16*). Results with fused heterocultures are shown, expressed relative to results with pPRL-CAT (−1957) after fusion, which yielded 9% conversion of [^{14}C]chloramphenicol to its acetylated products. In unfused heterocultures, expression of the constructs shown was undetectable (<0.1% of the expression of pPRL-CAT after fusion), except for prolactin constructs 5′Δ-204, 5′Δ-316, and 5′Δ-958, which exhibited 2.3, 18, and 0.9%, respectively, of the expression by pPRL-CAT after fusion. Expression of pPRL-CAT in a fused homogeneous C127 cell culture was also undetectable.

tor that binds to this region or some influence of the cotransfected Rous sarcoma virus promoter. Finally, direct transfer into pituitary cells yields expression of a growth hormone promoter construct, whereas transfection-fusion does not. It seems likely that these differences are due to either (or both) of the following properties of the transfection-fusion assay: (i) It should detect only those regulatory DNA sequences that interact with positive trans-acting factors. (ii) In this assay, pituitary cell factors interact with a prolactin promoter region previously chromatinized and silenced in fibroblasts during many generations of cell growth.

The transfection-fusion assay used in these experiments should be useful for similar studies of other transferred specialized genes, including those expressed specifically in cell types that are difficult to culture or are poor expressers of directly transferred DNA.

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8. Plasmid pRSV-NEO was constructed by replacing the Hind III–Bam HI CAT gene fragment of pRSV-CAT [C. M. Gorman, G. T. Merlino, M. C. Willingham, I. Pastan, B. H. Howard, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6777 (1982)] with the Hind III–Bam HI NEO gene fragment of pSV2NEO (*28*). Calcium phosphate coprecipitation [F. L. Graham and A. J. Van der Erb, *Virology* **52**, 456 (1973)] was used to cotransfect C127 cells [D. R. Lowy, E. Rands, E. M. Scolnick, *J. Virol.* **26**, 291 (1978)] with each promoter-CAT construct plus pRSV-NEO at a 20:1 DNA mass ratio. After 3 weeks of incubation with G418 (400 μg/ml active weight), polyclonal stably transfected cultures were formed by pooling 100 to 300 surviving clones. Southern blot gel analysis of the C127 cells transfected with pPRL-CAT or pGH-CAT showed that each construct was present in an apparently unrearranged form.
9. For fusion assays, 5×10^6 GH_3 cells from a suspension culture were seeded per 100-mm dish; the dishes had been treated with poly-L-lysine (10 μg/ml) to facilitate cell attachment. After 2 to 4 hours, 50% of a confluent polyclonal culture of stably transfected C127 cells (about 2×10^6 cells) was seeded onto the dish, which then incubated until the culture was visibly confluent (1 to 2 days). After two washes with phosphate-buffered salts lacking both Mg^{2+} and Ca^{2+} (PBS), dishes received 2.0 ml of a solution of polyethylene glycol 1000 (PEG) (Baker) pretreated as described [G. H. Yoakum, *BioTechniques* **2** (no. 1), 24 (1984)] and then dissolved in Dulbecco's modified Eagle's medium (DMEM). This solution was evenly distributed over the dish by rocking. The dishes were incubated 1.5 to 2.0 minutes at room temperature; the solution was then removed by aspiration. After four washes with PBS, the heterocultures received daily changes of DMEM containing 10% fetal calf serum. Fused cells were visible after 18 hours of incubation, and contained on average four nuclei per cell (nuclei recovered per cells recovered). Heterocultures were harvested 48 to 72 hours after fusion (in the plateau of CAT enzyme accumulation).
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11. Cellular levels and transcription start sites of specific mRNAs were assayed by ribonuclease protection of SP6-generated RNA probes [K. Zinn, D. DiMaio, T. Maniatis, *Cell* **34**, 865 (1983); D. A. Melton, P. A. Krieg, M. R. Rebagliati, T. Maniatis, M. R. Green, *Nucleic Acids Res.* **12**, 7035 (1984)], as described in detail elsewhere (*19*). Polyadenylated [poly(A)$^+$] RNA to be analyzed was prepared by poly(U)-Sepharose chromatography of cytoplasmic RNA. The Sst I–Eco RI PRL-CAT fragment from pPRL-CAT cloned into plasmid pSP65 was used to synthesize a 940-nucleotide ^{32}P-labeled antisense RNA, 302 nucleotides of which should be protected by correctly initiated PRL-CAT mRNA. CAT sense RNA was used as an internal quantitation standard and was synthesized from the pSV2CAT (*16*) Hind III–Mbo I CAT fragment. Incorporation of [^{3}H]uridine 5′-triphosphate was used to quantitate the RNA product, which should protect 255 nucleotides of the above antisense RNA. For analysis of mouse prolactin and growth hormone mRNA, the Hinc II–Pst I fragment from mouse prolactin complementary DNA (cDNA) and the Xho II–Pst I fragment from mouse growth hormone cDNA [D. I. H. Linzer and F. Talamantes, *J. Biol. Chem.* **260**, 9574 (1985)] were used to synthesize, respectively, the following ^{32}P-labeled antisense RNAs: 238- and 298-nucleotide probes, of which 189 and 232 nucleotides should be protected by, respectively, mouse prolactin and growth hormone mRNA. Probes for these mouse mRNAs did not hybridize to the corresponding rat mRNAs (Fig. 2, lanes 4 in panels B and C). The ^{3}H-labeled sense RNAs prepared as quantitation standards for mouse prolactin and growth hormone mRNAs should protect the entire length of the corresponding ^{32}P-labeled RNA probes. Results were quantitated by scanning.
12. The probes we used detected highly abundant transcripts of the corresponding mouse genes in total mouse pituitary RNA (T. Lufkin and C. Bancroft, unpublished data).
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14. The nuclear run-on transcription assay was per-

formed essentialy as described [M. E. Greenberg and E. B. Ziff, *Nature (London)* **311**, 433 (1984)], but with modifications described elsewhere (*19*). Each nitrocellulose filter "slot" contained 1.0 μg of denatured, immobilized DNA. After hybridization with a solution containing 10^7 count/min per milliliter of RNA sample, filters were washed and exposed to x-ray film for 13 days at −70°C with intensifying screen, and results quantitated by densitometry. The observation of a considerably more intense signal with a ribosomal RNA probe than with probes for mRNA transcripts (Fig. 3) demonstrates that for the latter, immobilized DNA was in excess.

15. The 5′-deletion mutants of pPRL-CAT and pGH-CAT were constructed as follows. To generate pPRL-CAT deletions containing 958 and 618 nucleotides, respectively, of prolactin 5′-flanking sequences, pPRL-CAT was digested with Sma I and then Sph I or Sst I, then treated to make the ends blunt and religated. Further 5′ deletions were prepared by Bal 31 digestion initiated in the polylinker of the −618 deletion. All deletions share the same vector sequences 5′ to the prolactin deletion end point. Deletion end points were determined precisely by dideoxy sequencing [E. Y. Chem and P. J. Seeburg, *DNA* **4**, 165 (1985)]. Plasmid pΔGH-CAT, with 0.236 kb of growth hormone–gene 5′-flanking sequence, contains the pGH-CAT Bgl II–Bam HI fragment cloned into the pUC 9 Bam HI site.
16. CAT activity was assayed essentially as described [C. M. Gorman, L. F. Moffat, B. H. Howard, *Mol. Cell. Biol.* **2**, 1044 (1982)], except that extracts were incubated at 65°C for 5 minutes to inactivate an endogenous deacetylase [M. Mercola, J. Goverman, C. Mirell, K. Calame, *Science* **227**, 266 (1985)]. Incubation was at 37°C for 8 to 12 hours, in the linear range of the assay. Increased levels of CAT enzymatic activity were detectable as early as 12 hours after cell fusion.
17. The following results imply that activation also does not require the 38 bp of prolactin gene body sequences present in these pPRL-CAT derivatives. Plasmid pPΔmCAT contains the prolactin DNA sequences between Pst I sites at −1957 and −11, linked through a Hind III linker at −11 to the Hind III site of the Hind III–Bam HI CAT gene fragment, cloned into pUC 9. In fused heterocultures, equal levels (*11*) of CAT mRNA were yielded by pPΔmCAT and pPRL-CAT.
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Three Recessive Loci Required for Insulin-Dependent Diabetes in Nonobese Diabetic Mice

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A polygenic basis for susceptibility to insulin-dependent diabetes in nonobese diabetic (NOD) mice has been established by outcross to a related inbred strain, nonobese normal (NON). Analysis of first and second backcross progeny has shown that at least three recessive genes are required for development of overt diabetes. One, *Idd-1*^{s}, is tightly linked to the *H-2K* locus on chromosome 17; another, *Idd-2*^{s}, is localized proximal to the *Thy-1/Alp-1* cluster on chromosome 9. Segregation of a third, *Idd-3*^{s}, could be shown in a second backcross. Neither *Idd-1*^{s} nor *Idd-2*^{s} could individually be identified as the locus controlling insulitis; leukocytic infiltrates in pancreas were common in most asymptomatic BC1 mice. Both F1 and BC1 mice exhibited the unusually high percentage of splenic T lymphocytes characteristic of NOD, suggesting dominant inheritance of this trait. The polygenic control of diabetogenesis in NOD mice, in which a recessive gene linked to the major histocompatibility complex is but one of several controlling loci, suggests that similar polygenic interactions underlie this type of diabetes in humans.

NONOBESE DIABETIC (NOD) MICE derived by Makino *et al.* from the non-inbred ICR strain are a model for insulin-dependent diabetes (Idd) in man (*1*). Both cellular and humoral autoimmunity against pancreatic β cells appears to be a central feature in pathogenesis (*2, 3*). Insulitis, a leukocytic infiltration of the pancreatic islets, is a salient histopathological lesion (*1, 4*). Insulitis was found at approximately 40% frequency in reciprocal [(NOD × NON)F1 × NOD] backcross mice at 9 weeks of age, suggesting control by a single recessive trait inherited from NOD mice (*4*). Subsequently, a diabetogenic recessive gene was associated with the unique NOD *H-2* haplotype on chromosome 17 (Chr 17) when an outcross/backcross analysis was performed between NOD and C3H mice (*5*). However, the low frequency of overt diabetes obtained in this analysis suggested the involvement of more than one NOD-derived recessive gene.

Breeding stock of the NOD strain and a related diabetes-resistant strain, nonobese normal (NON) were used at inbred generation F32 and F35, respectively. We have continued inbreeding and designate our sublines as NOD/Lt and NON/Lt; these inbred strains differ at numerous loci including the major histocompatibility complex (MHC), *H-2* (*4–6*). Consistent with the previously observed diabetes incidence of 80% in females and 10% in males (*1*), NOD/Lt females exhibit a diabetes incidence of >90% by 10 months of age; however, NOD/Lt males exhibit a higher than reported diabetes incidence, averaging 50 to 70% by 10 months when fed diet formulation 96W (Emory Morse Co., Guilford, Connecticut). F1 mice (23 males and 24 females) from reciprocal NOD/Lt × NON/Lt outcrosses were studied for development of hyperglycemia over a 12-month period. In contrast to the high incidence of hyperglycemia in parental NOD/Lt mice of both sexes by 12 months of age, F1 mice of both sexes were uniformly diabetes-resistant. NON/Lt can be distinguished from NOD/Lt mice by the number and functions of T lymphocytes in peripheral blood and spleen, as well as by their *Thy-1* phenotypes [Thy-1.1 and -1.2, respectively (6)]. NON/Lt mice develop T-lymphocytopenia associated with an age-dependent decline in responsiveness to concanavalin A whereas NOD mice show a persistent T cell hyperplasia associated with strong T cell mitogenic responses (*7*). However, the numbers and mitogen responsiveness of T lymphocytes in all F1 mice examined were NOD-like (*7*). Indeed, although pancreatic islet structure was intact and numbers of granulated β cells were normal, focal pancreatic lymphocytic infiltrates in perivascular and periductular areas, often abutting islets at one pole (but quite distinct from the insulitis observed in NOD parental mice), were noted in five of nine F1 males and nine of ten females examined histologically at 12 months. Thus, both recessive and dominant traits inherited from NOD were associated with pathogenesis.

In the present study, the genetic polymorphisms distinguishing NOD/Lt from

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