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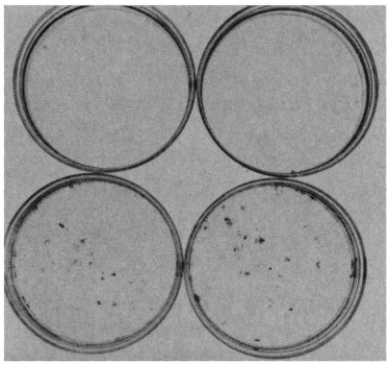

Fig. 1. Fat foci after transfection of DNA into 3T3-C2 cells. (**Top**) Transfection of uncut 3T3-C2 DNA in to 3T3-C2 cells. (**Bottom**) Transfection of uncut 3T3-F442A DNA into 3T3-C2 cells. Plated cells were treated with insulin (5 μg/ml) for 21 days and stained with oil red O.

Commitment of Mouse Fibroblasts to Adipocyte Differentiation by DNA Transfection

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Cells of the mouse cell line 3T3-F442A can be induced by various hormones to differentiate into adipocytes, whereas cells of 3T3-C2, a subclone of 3T3, cannot. However, transfection of DNA from uninduced 3T3-F422A cells into 3T3-C2 cells permits recovery of 3T3-C2 transfectants that differentiate into adipocytes in the presence of insulin. DNA isolated from human fat tissue, when transfected into 3T3-C2 mouse cells, also gives rise to mouse transfectants that are induced to differentiate into adipocytes by the addition of insulin. Apparently, transfection of a trans-regulatory gene (or genes) from 3T3-F442A or human fat cells into 3T3-C2 cells is sufficient to commit 3T3-C2 cells to adipocyte differentiation.

DIFFERENTIATION REQUIRES THE concerted expression of large numbers of structural and regulatory genes. Before entering a pathway of specific differentiation, a cell must be committed to that pathway. Commitment, or the capacity to become differentiated, can precede the differentiation itself by a considerable time. Committed cells require an inducer or inducers to initiate the cascade of biochemical events that result in a differentiation.

The capacity to introduce DNA from a committed but undifferentiated cell into an uncommitted recipient cell offers a straightforward strategy for detection and eventual isolation of a committing gene for that differentiation pathway. Lassar *et al.* (*1*) showed that DNA from 5-azacytidine–treated myoblasts can induce myoblast differentiation in a culture of mouse fibroblasts; the DNA of the cells of the donor must first be demethylated by 5-azacytidine for differentiation to occur on transfection. They identified a cDNA clone from committed myoblasts that, when transfected into fibroblast cells, is sufficient to convert them to stable myoblasts (*2*). Pinney *et al.* (*3*) passaged cosmid vectors containing the human genomic library and a selectable drug resistance gene through methylase-deficient bacteria to obtain hypomethylated and thus potentially active DNA; stable myogenic cell lines were isolated when this DNA was transfected into the multipotential C3H/10T½ mouse embryo cell line.

We studied the pathway of adipocyte differentiation. When the growth of 3T3 mouse fibroblasts is arrested in culture, a minority of susceptible fibroblasts express the adipocyte phenotype (*4, 5*). Expression is accelerated by long-term exposure of the cells to insulin or brief treatment of the cells at confluence with dexamethasone and methylisobutylxanthine (*4–7*). Induced and fully differentiated adipocytes derived from 3T3 cells show the biochemical characteristics and hormone responsiveness found in true fat tissue adipocytes (*8–19*). By cloning, Green and Kehinde (*4*) isolated several 3T3-derived preadipocyte cell lines, such as 3T3-L1 and 3T3-F442A, which convert to adipocytes with almost 100% efficiency in the presence of high levels of insulin. They also obtained sublines of 3T3, such as 3T3-C2, that have a small frequency of spontaneous fat conversion and cannot be induced to adipocyte differentiation even by high levels of insulin (*5*). Nixon and Green (*19*) reported the frequency of 3T3-C2 adipogenesis to be less than 1% of 3T3-F442A. In our experiments 3T3-C2 cells were not inducible: we detected no adipocytes in $>10^8$ insulin-treated 3T3-C2 cells.

We isolated high molecular weight genomic DNA from untreated 3T3-F442A cells and cotransfected it with a plasmid carrying a neomycin (G418) resistance gene into 3T3-C2 recipient cells. After about 2 weeks in selective G418 medium (400 μg/ml), many cells survived. Most did not form large colonies, but about ten visible G418-resistant colonies (~100 cells each) were found on each plate together with about 10^3 cells, separate and in small clusters. All survivor cells were pooled and replated onto a fresh plate. As replated survivors grew into a confluent monolayer, they were treated with insulin. The plates that survived the insulin treatment were then stained with oil red O (Fig. 1). Although the number of fat foci varied greatly from monolayer to monolayer, it is clear that 3T3-F442A DNA, but not 3T3-C2 DNA, conferred on 3T3-C2 cells the capacity to differentiate into adipocytes (Table 1).

Only cells that had taken up DNA sur-

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vived in the selective medium containing G418. To get a confluent culture for insulin treatment, we trypsinized and pooled survivors. Because these survivors had not always grown into visible colonies, it was difficult to estimate the number of 3T3-C2 cells that had taken up DNA successfully. We detected no conversion when we tried to induce the separate unpooled G418-resistant colonies to adipogenesis with insulin in order to quantitate the adipocyte conversion frequency. Apparently, cell-cell confluence is a prerequisite for adipocyte differentiation in recipient 3T3-C2 cells, as it is in donor 3T3-F442A cells (*5, 6*). Thus we have not yet quantitated the efficiency of conversion of 3T3-C2 cells per microgram of 3T3-F442A DNA. Nevertheless, the occurrence of fat foci in replated G418-resistant 3T3-C2 cells after transfection with 3T3-F442A DNA is reproducible.

As 3T3-F442A cells differentiate, specific activity of the enzyme glycerol phosphate dehydrogenase increases about 1000-fold, from a baseline of 3 units per milligram of protein (1 unit of enzyme activity corresponds to the oxidation of 1 n*M* of NADH per minute) (*10, 11*). In cultures of 3T3-C2 cells replated after transfection with 3T3-F442A DNA, we obtained, on average, 20 to 30 foci per 10^6 cells in a confluent 60-mm plate (Table 1). Since each fat focus contains 50 to 100 cells, no more than 10^3 cells of the 10^6 in the dish were likely to have contributed to the overall increase in enzyme activity of the culture. One thousand differentiating cells, each showing a 10^3-fold increase in activity, would generate about a twofold increase in the specific activity of 10^6 pooled cells. In several experiments we observed two- to fourfold increases in glycerol phosphate dehydrogenase activity in 3T3-F442A DNA–transfected 3T3-C2 cells, when compared to the activity seen in 3T3-C2 cells transfected with 3T3-C2 DNA.

If the committing activity of 3T3-F442A DNA is contained in a specific DNA sequence, then in some cases restriction enzyme digestion of 3T3-F442A DNA should abolish the ability of high molecular weight genomic DNA from 3T3-F442A to convert 3T3-C2 cells to adipocytes. We tried four different restriction enzymes: Bam HI, Eco RI, Hind III, and Kpn I. Each of the enzymes recognizes sequences of six bases long, so on a random basis each enzyme would be expected to cleave once for every 4×10^3 base pairs. Portions of high molecular weight 3T3-F442A DNA were digested to completion separately with each enzyme. Each digested DNA was then cotransfected into 3T3-C2 cells with the neomycin resistance gene as the selectable marker. About 2 weeks after transfection, G418-resistant cells were pooled and allowed to become confluent before treatment with insulin. Three to four weeks later the plates were fixed and stained with oil red O. Control 3T3-C2 DNA was also subjected to digestion with the same set of restriction enzymes and transfected into 3T3-C2 cells.

Digestion of 3T3-F442A DNA with restriction enzymes Bam HI, Eco RI, and Kpn I did not change its ability to commit 3T3-C2 cells to adipocytes (Table 1). The number of induced fat foci observed with uncut 3T3-F442A DNA was similar to that of 3T3-F442A DNA digested with any of these enzymes (Table 1). However, digestion of 3T3-F442A DNA with the restriction enzyme Hind III markedly reduced the adipocyte conversion of 3T3-C2 cells (Table 1). In control experiments, no preparation

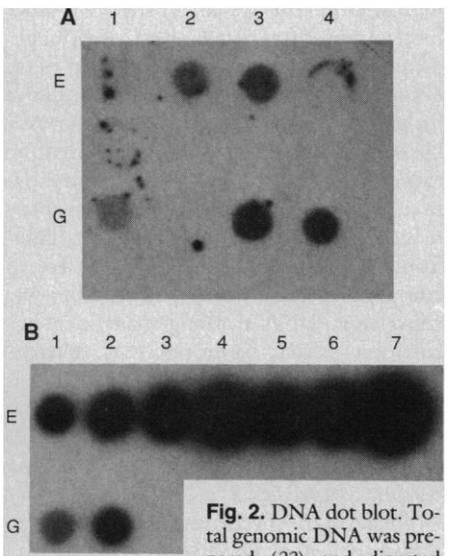

Fig. 2. DNA dot blot. Total genomic DNA was prepared (*22*) and digested with Bam HI. Each DNA sample was denatured in 0.25*N* NaOH then loaded onto GeneScreen Plus in a dot blot manifold. The GeneScreen Plus was then probed with ^{32}P-labeled agarose gel–purified DNA fragments containing either the neomycin resistance gene or the human Alu sequences (*25*). (**A**) Alu probe. Sections E1 to E3, agarose gel–purified Alu DNA fragment added at 1, 10, and 25 pg to 20 μg of Bam HI–digested 3T3-C2 mouse DNA; G1, 20 μg of C2C2A, a secondary clone of 3T3-C2 cells transfected with 3T3-C2 DNA; G3, a subclone of secondary transfectant of C2/Fo46-1 at 20 μg; and G4, secondary transfectant C2/Hufat-1a at 20 μg. Human repetitive Alu sequences are seen in C2/Fo46-1 and C2/Hufat-1a. E4 and G2 are blank. (**B**) Neomycin probe. Sections E1 to E7 agarose gel–purified neomycin DNA fragment at 1, 3, 5, 10, 15, 25, and 50 pg; G1, 20 μg of DNA from secondary transfectant C2/Fo46-1; and G2, 20 μg of a subclone of C2/Fo46-1. G3 is blank. All clones showed integrated neomycin DNA as expected.

Table 1. Adipocyte differentiation in 3T3-C2 cells induced by insulin after DNA transfection. 3T3-C2 and 3T3-F442A cells were grown in Dulbecco's modified Eagle's medium plus 10% calf or fetal calf serum (Gibco). High molecular weight DNA was isolated as described (*22*). DNA size was determined on 0.4% agarose gel. Restriction enzymes were used according to the vendor's instruction (New England Biolabs). Digested DNA was analyzed on agarose gels to assure complete digestion. The calcium phosphate transfection procedure was essentially the same as described previously (*23*). The pKoNeo plasmid was used to cotransfect a selectable marker (*24*), resistance to G418 (Gibco). The lowest concentration of G418 that kills all 3T3-C2 cells in a 2-week period is 400 μg/ml. Cells surviving G418 (400 μ/ml) for 2 weeks, in both colonies and small cell clusters, were trypsinized and replated in medium without G418. When the replated cells had grown to confluence, at about 7 days, insulin (Sigma) (5 μg/ml) was added. In about 85% of the plates containing cells transfected with either type of DNA, induction with insulin led to rapid growth and formation of a sheet, which then peeled off the plates within 3 to 5 days. The cells in the rest of the plates responded to insulin induction by establishing a stable monolayer. Only the cells in monolayers could be tested for their response to insulin induction by becoming adipocytes. In positive cultures, adipocyte foci were seen against a background of confluent undifferentiated C2 cells after 30 days in insulin. Plates were fixed with formaldehyde and stained with oil red O. Fat foci were scanned under a dissecting microscope at ×16 or ×40 after oil red O staining. Each fat focus was found to contain at least 50 fat cells. Data are presented as foci per 60-mm plate. The total number of plates are from 11 independent experiments. Neomycin resistance, Neo^r.

Donor DNA	Average number of Neo^r colonies per plate (±SD)	Total number of plates induced by insulin	Total number of plates surviving treatment with insulin	Average number of fat foci per surviving plate (±SD)	Range of fat foci per surviving plate
3T3-F442A	12.3 ± 1.2	459	67	26.8 ± 20	2 to 72
3T3-C2	14.2 ± 0.9	423	65	0	0
3T3-F442A/ Bam HI	12.1 ± 0.8	90	14	31.9 ± 16	4 to >60
3T3-F442A/ Eco RI	11.4 ± 1.1	94	17	22.0 ± 18	2 to >60
3T3-F442A/ Kpn I	13.5 ± 0.4	103	18	28.5 ± 16	6 to >60
3T3-F442A/ Hind III	12.1 ± 1.2	90	19	0.18 ± 0.4	0 to 1
3T3-C2/ Bam HI	14.1 ± 1.0	92	13	0	0
3T3-C2/ Eco RI	14.1 ± 0.9	91	15	0	0
3T3-C2/ Kpn I	13.9 ± 1.3	101	14	0	0
3T3-C2/ Hind III	10.3 ± 1.1	98	12	0	0

of 3T3-C2 DNA (cut or uncut) gave rise to any adipocyte conversion after transfection into 3T3-C2 cells.

Fully differentiated adult human fat cells also contain DNA sequences that can commit 3T3-C2 cells to adipocyte differentiation. High molecular weight DNA was isolated from biopsied human fat (Fo46, Fo95, Fo69, and Hufat-1) and co-transfected with G418-resistant plasmid into 3T3-C2 cells. DNA from cultured human skin fibroblasts (NHF) was prepared as a control. Neomycin-resistant surviving cells were pooled and grown. DNA from the primary transfectants was then used as donor DNA for secondary transfection into 3T3-C2 cells, without additional plasmid containing the neomycin resistance gene. Four secondary G418-resistant colonies from 3T3-C2/Fo46 DNA were isolated by neomycin resistance, replated, and treated with insulin at confluence. Two of the induced secondary clones (3T3-C2/Fo46-1 and 3T3-C2/Fo46-3) showed fat foci (Table 2). Another secondary neomycin-resistant clone, C2/Hufat-1a, gave rise to fat foci, whereas DNA from 3T3-C2/Fo69 and 3T3-C2Fo95 cells did not confer adipocyte differentiation on secondary clones of 3T3-C2 cells. Thus, DNA isolated from human fat can convert mouse fibroblasts into preadipocytes that can be induced to differentiate into adipocytes.

We have begun to isolate the human DNA sequences transferring commitment to adipogenesis. The human repetitive Alu sequence was detected in secondary transfectants of human fat DNA. Using DNA dot blots, we were able to detect 1 to 10 pg of Alu per 20 μg of total DNA (Fig. 2A). DNA dot blots detected the neomycin resistance gene at 1 to 3 pg per 20 μg of genomic DNA (Fig. 2B). Since secondary clones were isolated by their resistance to neomycin and no additional neomycin plasmid was added in the secondary transfection, the human sequences responsible for the adipocyte differentiation are likely linked to the neomycin resistance gene in the secondary transfected lines.

RNA isolated from 3T3-C2/human fat secondary transfectant clones was examined by Northern blot for mRNA of enzymes involved in lipogenesis. Probes included the cDNA of malic enzyme, adenosine triphosphate (ATP) citrate lyase, and aP_2, the myelin P_2–like protein. We detected aP_2 mRNA in RNA isolated from insulin-induced 3T3-C2/Fo46-1-9A, a subclone of the secondary transfectant, at a level of about 1/50 to 1/100 of that in RNA from human fat tissue. No aP_2 mRNA was detected in 3T3-C2 nor in a 3T3-C2 clone transfected with 3T3-C2 DNA.

A relatively small, specific DNA sequence in the 3T3-F442A genome is capable of conferring the commitment to adipocyte conversion on 3T3-C2 cells. Because conversion requires the concerted expression of 40 to 60 structural genes (*7, 8, 10–19*), we hypothesize that we have transfected a sequence that is required for the commitment to adipocyte differentiation, rather than one encoding any of the structural genes activated during induction of a committed preadipocyte.

Treatment of 3T3-C2 cells with 5-azacytidine did not commit 3T3-C2 cells to the adipocyte pathway. Thus we have no reason to expect methylation to be an operative mechanism in this system; at least two other mechanisms may be involved. Rearrangement of DNA in the variable region of an immunoglobulin light chain is a prerequisite for production of antibodies (*20*). It is possible that 3T3-F442A cells have acquired the capacity to convert to adipocyte differentiation because their DNA has been rearranged. If so, 3T3-C2 cells would differ from 3T3-F442A cells in being unable to rearrange their DNA to permit adipocyte conversion. DNA rearrangement in differentiation is likely to be rare (*21*). Alternatively, 3T3-F442A cells and human fat cells may donate, through transfection, a region of DNA that has been deleted in 3T3-C2 cells or that is inactive in cells that cannot become adipocytes.

Whatever the mechanism that provides an active sequence in 3T3-F442A cells and human fat tissues but not in 3T3-C2 cells, we have transfected DNA for adipocyte conversion from 3T3-F442A cells and from human fat cells into 3T3-C2 cells. This gene or its product is then capable of activating the cascade of adipocyte conversion in 3T3-C2 cells.

Table 2. Fat foci induced from secondary transfected 3T3-C2 cells. Human fat tissues were broken into small pieces with a mortar and pestle in the presence of liquid nitrogen. The small pieces of tissues were then homogenized in HB buffer (0.15*M* NaCl, 0.1*M* EDTA, and 0.05*M* boric acid, *p*H 9.6) and strained through gauze. SDS was added to final concentration of 2%; an equal volume of tris-saturated phenol was added and the mixture was left on a shaker overnight. The aqueous phase was then collected after 15 min of centrifugation at 2200*g*. After ethanol precipitation, we treated the mixture with ribonuclease A for 1 hour at 37°C. After ribonuclease A incubation, SDS was added to 0.5% and proteinase K was added to 0.5 mg/ml, and the mixture was incubated for 2 hours at 37°C. The sample was then extracted at least three times with phenol-chloroform-isoamyl alcohol (25:24:1) before ethanol precipitation. Each sample was checked by 0.4% agarose gel electrophoresis to assure high molecular weight DNA had been obtained. Transfection into 3T3-C2 cells was as described in Table 1. Primary neomycin-resistant clones were pooled and grown for isolation of DNA to use in secondary transfection. The donor DNAs 3T3-C2/Fo46, 3T3-C2/NHF, 3T3-C2Hufat-1, 3T3-C2/Fo95, and 3T3-C2/Fo69 are from primary transfectants. Secondary transfection was done without additional neomycin plasmid. Secondary neomycin-resistant colonies were cloned, replated and treated with insulin (5 μg/ml) for 4 to 6 weeks before staining with oil red O. Data are given as the means ± SD.

Donor DNA	Secondary neo^r 3T3-C2 colonies	Fat foci per plate
3T3-C2/Fo46	3T3-C2/Fo46-1	10.7 ± 5.8
	3T3-C2/Fo46-2	0
	3T3-C2/Fo46-3	3.6 ± 2.6
	3T3-C2/Fo46-4	0
3T3-C2/NHF	3T3-C2/NHF-1	0
	3T3-C2/NHF-2	0
	3T3-C2/NHF-3	0
	3T3-C2/NHF-4	0
	3T3-C2/NHF-5	0
3T3-C2/Hufat-1	3T3-C2/Hufat-1a	16.8 ± 13.1
	3T3-C2/Hufat-1b	0
	3T3-C2/Hufat-1c	0
	3T3-C2/Hufat-1d	0
3T3-C2/Fo95	3T3-C2/Fo95-1	0
	3T3-C2/Fo95-2	0
	3T3-C2/Fo95-3	0
	3T3-C2/Fo95-4	0
	3T3-C2/Fo95-5	0
	3T3-C2/Fo95-6	0
3T3-C2/Fo69	3T3-C2/Fo69-1	0
	3T3-C2/Fo69-2	0

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Synaptic Connections in Vitro: Modulation of Number and Efficacy by Electrical Activity

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The functional architecture of synaptic circuits is determined to a crucial degree by the patterns of electrical activity that occur during development. Studies with an in vitro preparation of mammalian sensory neurons projecting to ventral spinal cord neurons show that electrical activity induces competitive processes that regulate synaptic efficacy so as to favor activated pathways over inactive convergent pathways. At the same time, electrical activity initiates noncompetitive processes that increase the number of axonal connections between these sensory and spinal cord neurons.

PATTERNS OF NEURONAL ELECTRICAL activity play an important role in determining the development of appropriate functional synapses, as has been shown in studies on the vertebrate visual cortex (*1*). Activity-dependent changes in synaptic organization occur in many areas of the nervous system in response to sensory deprivation (*2*) or experimental regulation of neural activity (*3*). Data suggest that multiple afferents converging on a common target interact competitively, resulting in the weakening or elimination of "inappropriate" connections and the maintenance of functionally effective afferents (*4*). "Appropriate" connections are those provided from sets of neurons that fire so close to synchronously that they produce a critical state of activation of the target neuronal population (*5*).

In the present studies, part of the circuitry from the developing spinal cord was reconstructed in vitro, which made it possible to manipulate activity from sensory neurons to spinal cord neurons and measure intracellularly the strength of synapses from competing inputs. Neurons dissociated from 13-day fetal mouse spinal cords were cultured in a three-compartment tissue culture chamber (*6*) (Fig. 1). After approximately 1 week in culture, axons from dorsal root ganglion (DRG) neurons plated in the two side compartments penetrated the thin fluid space under the barrier to form synapses on ventral horn (VH) spinal neurons in the central compartment. Horseradish peroxidase injection and electrophysiological investigation gave no evidence of DRG projection from one side compartment to the other in these experiments. Nine to 12 days after the DRG neurons were plated, a phasic pattern of electrical stimulation was applied to the axons extending from one of the side compartments (*7*). The stimulation did not produce any change in the *p*H of the culture medium and had no substantial effect on DRG survival. The electrode for the center compartment was more than 1 cm removed from the VH neurons in the center slot. For these reasons we conclude that the stimuli were not producing nonspecific physicochemical effects on the system. After 3 to 5 days of stimulation, intracellular recordings were obtained from VH neurons to determine the number of functional inputs and the amplitudes of excitatory postsynaptic potentials (EPSPs) generated by axons from each side (Fig. 2A) (*8*). Control preparations were treated similarly except that neither side chamber was stimulated. VH neurons were selected from random locations in the central compartment, and the experimenter was not aware of the stimulus condition at the time of recording.

The protocol results in three categories of afferents to VH neurons: (i) stimulated afferents, (ii) nonstimulated convergent afferents, and (iii) afferents from controls in which neither side compartment was stimulated. Long-term stimulation of the DRG afferents affected the EPSPs significantly. In unilaterally stimulated chambers, the median EPSP produced by the long-term stimulation of the DRG axon was 10.0 mV, whereas the median EPSP produced by the nonstimulated convergent DRG axons was 3.9 mV ($P < 0.03$, Mann-Whitney U test). In control chambers, this value was intermediate at 5.5 mV (Table 1). This data set shows that stimulation affects EPSP amplitudes with a significance level of $P < 0.005$ based on the Kruskal Wallace test (*9*).

The change in synaptic strength could be due to a change in the number of axons that establish functional connections with the target VH neuron. To investigate this ques-

Table 1. The strength of synapses that form during development is influenced by differences in the level of electrical activity between axons that converge on the same neuron. The strength of synapses was increased in afferents exposed to a phasic pattern of stimulation during development, but the amplitude of EPSPs from inactive afferents was reduced below that of controls in which neither side compartment was stimulated.

	Amplitude of EPSPs evoked in VH neurons by DRG axon stimulation (mV)				
	Median	Mean rank	Mean	SD	No. of sides
Controls (no stimulation)	5.49	128	11.7	13.11	108
Stimulated side	10.00*	149	15.8	14.55	79
Nonstimulated side	3.89	122	10.5	13.37	77

*$P < 0.03$, Mann-Whitney U test, versus nonstimulated side.

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