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Genomic sequences capable of committing mouse and rat fibroblasts to adipogenesis

Luz Colón-Teicher, Leigh S.Wise, Jeffrey J.Martino, Leonard Baskin+, George Sakoulas, Robert E.Pollack and Suzie Chen*
Department of Biological Sciences, Columbia University, New York, NY 10027, USA

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ABSTRACT

The mouse Swiss 3T3-F442A/3T3-C2 cell system is well suited for the isolation of genes involved in commitment to adipogenesis. 3T3-F442A cells convert to adipocytes with high efficiency in response to confluence and insulin. The sister clonal line 3T3-C2 does not respond to these signals, but can convert to adipocytes when transfected with DNA from 3T3-F442A preadipocytes or from human fat. Human fat-tissue biopsy FO46 DNA transfected into 3T3-C2 gave rise to fat foci after two rounds of transfection and selection. A cosmid library of a subclone of secondary transfectant 3T3-C2/FO46-1 was screened for the human repetitive Alu sequence. Five out of eight Alu + recombinant clones committed 3T3-C2 cells to adipogenesis. The adipose commitment (AC) activity of one cosmid, p18A4, was found to reside in two small, non-identical, subcloned sequences 1.2kb and 2.0kb in length, each separately able to commit 3T3-C2, precrisis mouse and rat fibroblasts and the multipotential C3H10T1/2 cell line to adipogenesis. We conclude that commitment to adipogenesis can be effected *in vitro* with high efficiency by transfection of specific sequences into a variety of host cells.

INTRODUCTION

Early in the development of higher organisms, cells become determined or committed to a specific differentiation pathway. The committed state is characteristically preserved through a variable number of cell divisions until the proper set of environmental signals induce irreversible, terminal differentiation. Although it is clear that specific genes are involved in the commitment process (1,2), the regulatory factors and molecular events that give rise to committed cells in the embryo are largely unknown.

Transfer of genetic material into recipient cells by transfection has been used successfully to isolate and characterize genes responsible for particular phenotypic traits. Genes involved in commitment, if they exert a dominant effect, can be isolated and cloned by transfection of DNA from committed cells into appropriate uncommitted cells. The Swiss 3T3-derived F442A and C2 cell lines provide a system suitable for the isolation, by transfection, of genes involved in commitment to adipogenesis (3–5). During growth these fibroblastic cells are indistinguishable from one another. 3T3-F442A preadipocytes convert to adipocytes with high efficiency in response to confluence, and the conversion is accelerated by the presence of high levels of insulin. In contrast, 3T3-C2 cells do not respond to either confluence or insulin; in our laboratory these cells have no detectable frequency of conversion with more than 10^8 cells examined.

We have shown previously that 3T3-C2 cells are able to convert to adipocytes upon insulin stimulation at confluence when successfully transfected with high molecular weight DNA extracted from 3T3-F442A preadipocytes (5). Secondary transfectant clones isolated from 3T3-C2 cells that have taken up human fat tissue DNA (biopsy FO46), can also convert to adipocytes. 3T3-C2 cells transfected with their own DNA failed to produce fat foci (5).

Now we describe the isolation of five cosmids capable of committing 3T3-C2 cells to adipogenesis, from a genomic DNA library of secondary transfectant subclone 3T3-C2/FO46-1-9A. We randomly chose one of the five cosmids, p18A4, to further analyze. The activity of this cosmid is mapped to two small, non-identical, subcloned sequences each of which is independently capable of committing mouse and rat fibroblasts to adipogenesis upon confluence and insulin induction. The behavior of preadipocytes induced by either one of these two subcloned sequences does not differ phenotypically from the behavior of the preadipocyte line 3T3-F442A.

MATERIALS AND METHODS

Cell cultures and AC assays

All cells were grown in DME medium plus 10% calf serum (Gibco BRL). High molecular weight DNA was isolated as described (6). Recombinant cosmid and plasmid DNAs were prepared as described (7). DNA digests were performed according to vendor's instructions (NE Biolabs); complete digestion was verified by agarose gel electrophoresis, and the

* To whom correspondence should be addressed at: Department of Chemical Biology & Pharmacognosy, College of Pharmacy, Rutgers University, Piscataway, NJ 08854, USA

+ Present address: Institute of Cancer Research, Columbia University, New York, NY 10032, USA

restricted DNAs were ethanol-precipitated after purification by phenol/chloroform extraction. DNAs to be tested were transfected into recipient cells together with pKoNeo as a selectable marker, except for unrestricted cosmid DNAs which contain the Neo^r gene in the vector. DNA transfections were performed by the calcium phosphate method modified as previously described (8). Sixty mm plates were seeded with recipient cells at a density of 4×10^5. Ten to twenty μg of DNA were transfected or co-transfected with pKoNeo. Cells taking in exogenous DNA were selected in medium containing G418 (Gibco) at the lowest concentration that kills untransfected cells in a 2-week period as experimentally determined (400 μg/ml for 3T3-C2 cells). Cells surviving G418 for 2–3 weeks were trypsinized and replated in medium without G418 and grown to confluence (approx. 7–10 days). This medium was then replaced with medium containing 5% FCS + insulin (5μg/ml) for adipocyte conversion. In MSF (C57L primary mouse skin fibroblasts) transfections individual G418^r colonies were expanded and split into sister cultures. Frozen sister cultures were thawed and retested. In positive cultures, adipocyte foci are seen against a background of confluent, undifferentiated cells. Three weeks after the first adipocytes are seen under phase contrast, plates were fixed with formaldehyde and stained with Oil-red-O. Fat foci were scanned under a dissecting microscope at ×16 or ×40 after staining and counted as positive if containing at least 50 fat cells.

Construction and screening of cosmid library

High molecular weight DNA (~200 kb) was extracted from the pooled 9A secondary transfectant fat cells as described (9). The DNAs were partially digested with *Mbo*I. 30–40 kb fragments were selected by sucrose gradient and ligated into the *Bam*HI site of phosphatase-treated cosmid vector pWE15 (Stratagene) at a 1:5 insert:vector molar ratio. The ligated DNAs were packaged using Gigapack Gold (Stratagene) mixtures and transduced into *E.coli* strain AG-1. Cells were plated onto 100 mm LB agar plates containing 50 μg/ml ampicillin (LB + Amp), at a density of 5×10^3 colonies/plate, and cultured overnight. The colonies were replicated onto duplicate nitrocellulose filters (Millipore) as described (7). One set of filters was for storage and the other for screening by the method of Grunstein and Hogness (10). Cells in the original plate were regenerated by culturing an additional 2–4 hr, then stored at 4° and used to obtain positive colonies after screening. Human *Alu* sequence for probe was obtained by PCR amplification of the *Alu* insert of pBlur-8 (11) primed by two 21-bp oligomers covering the ends of the *Alu* sequence. The probe was labeled to a specific activity of approximately 10^9 cpm/μg DNA by random primer extension (12) and used at a concentration of $2-5\times10^6$ cpm/ml.

Isolation of *Alu*+ cosmids

Areas of the plates corresponding to the autoradiograph's positive signals were selected and purified to single colonies by repeated replating and screening. Finally, to insure purity and stability, these single colonies containing *Alu* were re-spread on plates and re-screened. Stably *Alu*+ colonies were grown overnight in LB + Amp and frozen for further study.

Subcloning of p18A4 BamH1 fragments into pUC8

*Bam*H1-restricted p18A4 DNA fragments were electrophoresed in 0.8% agarose and electroeluted using Spectra/por 12–14,000 dialysis membrane as described (7). DNAs were purified through NACS Prepac columns (BRL) and ligated to *Bam*H1-cut, calf intestinal phosphatase-treated (Boehringer Mannheim) pUC8 vector DNA using T4 DNA ligase (NE Biolabs), 2.5 Weiss units/μg DNA, at a molar ratio of insert:vector >2. The ligated DNAs were used to transform *E.coli* strain XL-1 Blue (Stratagene). Well separated recombinant colonies were picked for DNA minipreps (7).

Southern blot hybridization

High molecular weight DNA from cultured cells was purified and digested with restriction enzymes (NE Biolabs) to completion. Five to ten micrograms of DNA per lane were run through a 0.8% agarose gel, transferred to either Genescreen Plus (DuPont) or Immobilon-N membrane (Millipore) and hybridized overnight to Clone A or B gel-purified or PCR-amplified insert. The probe was ^{32}P-labeled by random priming (12) to specific activity $\sim8-12\times10^8$ cpm/μg DNA. High stringency hybridization and washing conditions were followed as suggested by Manufacturer.

RESULTS

Isolation of cosmid clones with adipose commitment (AC) activity

3T3-C2/FO46-1-9A or simply 9A, a subclone of human secondary transfectant 3T3-C2/FO46-1, cells were shown to have the human repetitive *Alu* sequence by dot blot analysis confirming that human DNA had been successfully transferred through two transfections (5). A genomic library of 4×10^5 cosmids was assembled with high molecular weight DNA from 9A cloned into vector pWE15, for screening with radiolabeled human *Alu* sequence. One genome equivalent of the library yielded several *Alu*-positive signals; corresponding areas were purified to single *Alu*+ colonies as described in Methods, and tested for AC activity in 3T3-C2 cells. DNA from five out of eight cosmid clones was able to convert 3T3-C2 cells to adipocytes (Table 1). In positive transfectants, fat cells were detected 2–3 weeks sooner after insulin induction than when genomic 3T3-C2/FO46 DNA was used as donor, and in individual plates which contained fat foci, three- to four-fold more fat foci appeared with positive cosmid DNAs than with genomic DNA (Table 1).

We randomly chose one of the five AC-positive cosmids, p18A4, for further study. In order to further define the region of AC activity, p18A4 DNA was digested to completion with *Not*I, *Eco*RI, *Bam*HI and *Hind*III, and the restricted fragments were transfected into 3T3-C2 cells. *Hind*III noticeably reduced AC activity, while *Not*I-, *Eco*RI- or *Bam*HI-digested DNAs had no significant effect relative to that of the uncut cosmid. Southern analysis using pWE15 vector as probe showed that the largest *Bam*HI fragment of insert DNA in p18A4 was approximately 6 kb, indicating that at least some sequences capable of conferring adipogenic commitment could not exceed 6 kb in this cosmid.

Isolation of two pUC subclones with AC activity

Ten different *Bam*HI fragments of p18A4 were subcloned into pUC8 and co-transfected with pKoNeo into 3T3-C2 cells. The *Alu* sequence in p18A4 was contained in a single 300 bp *Bam*HI fragment which tested negative in our AC bioassay, as did all but two of the other pUC subclones. The two AC-positive pUC subclones contained small inserts from p18A4: one of 1.2 kb (Clone A) and the other of 2.0 kb (Clone B). By restriction, both contained an internal HindIII site. Clones A and B both hybridized to a single 12 kb *Eco*RI band of p18A4, but failed to hybridize

Table 1.

DONOR DNA	Recipient cells	First foci observed (week)	Average number of fat foci/ total Neo^r plates Mean ± S.D.	Range of fat foci/ plate	positive plates/ total Neo^r plates
Genomic					
3T3-C2	3T3-C2	–	0	0	0/65
C2/F046	3T3-C2	5	2.8 ± 2.8	1–22	19/29
Alu$^+$ Cosmid clones					
	p1B5	3T3-C2	3	9.3 13/2442	1–43
p18A4	3T3-C2	3	12.7 ± 15.6	1–84	18/44
p18D4	3T3-C2	–	0	0	0/18
p8C1	3T3-C2	3	8.7 ± 8.3	2–42	9/13
p8D1	3T3-C2	–	0	0	0/46
p27D1	3T3-C2	3	7.5 ± 7.4	1–39	24/36
p21A1	3T3-C2	3	9.7 ± 13.4	1–212	35/68
p21A4	3T3-C2	–	0	0	0/36
AC$^+$ p18A4 subclones and control					
Clone A	3T3-C2	1	144.5 ± 200.4	2– $>10^3$	16/32
Clone B	3T3-C2	1	164.5 ± 221.5	3– $>10^3$	16/31
pBLUR-8	3T3-C2	–	0	0	0/26
pWE15	3T3-C2	–	0	0	0/11
pKoNeo	10T1/2	–	0	0	0/21
Clone A	10T1/2	1	129.8 ± 158.6	3– $>10^3$	32/38
Clone B	10T1/2	1	259.5 ± 282.3	4– $>10^3$	24/29
pKoNeo	MSF	2	1.4 ± 1.5	1–6	6/12
Clone A	MSF	1	163.6 ± 251.3	9– $>10^3$	24/69
Clone B	MSF	1	143 ± 201.7	3– $>10^3$	14/17
pKoNeo	F2408	3	0.4 ± 0.6	1–3	3/12
Clone A	F2408	3	2.6 ± 2.5	1–12	14/24
Clone B	F2408	3	17 ± 16.5	3– $>10^2$	19/24

Recombinant cosmid clones plB5, pl8A4, p18D4, p8C1, p8D1, p27D1, p21A1 and p21A4 were purified from the 9A cosmid library as described in Methods. Donor DNAs were co-transfected into recipient cells with neomycin-resistance plasmid pKoNeo, except for cosmid clones which contain a ligated neomycin resistance gene. Cells surviving G418 (400 μg/ml) for 2–3 weeks were trypsinized and replated in fresh plates. The plates were treated at confluence with insulin (5μg/ml) for adipocyte conversion. Three weeks after the first adipocytes were seen, 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. The number of foci of at least 50 fat cells each were recorded. An example of the scatter of the number of fat foci on Neo^r plates, Clone B into 3T3-C2: 649, 412, 446, 65, 96, 5, 193, 3, 487, 515, 97, $>10^3$, 4, 115, $>10^3$, 12 and 15×0's. The average number of fat foci is the total number of fat foci over the total number of Neo^r plates. In co-transfection of PkoNeo plasmid and Clone A or B, 34% or greater of the pooled Neo^r plates were positive for adipocytes. A similar distribution of positive and negative dishes was observed by others in co-transfections of drug resistance and MyoD1 (25,26), presumably because some Neo^r cells fail to integrate the second donor DNA in a functional state. In fact, based on the present data and those presented by others (25, 26), less than half of the Neo^r plates have both types of DNA.

to each other, or to the inserts of the other active cosmids derived from the same library (data not shown).

Clones A and B both bring about conversion to adipocytes independently and with similar efficiency (Table 1). Both clones induced fat foci as early as one week post confluence, a time course much like 3T3-F442A preadipocyte cells, and the maximum number of fat foci per dish was $>10^3$. These kinetics are more rapid than those of cells transfected with p18A4 cosmid DNA, but similar to those of 3T3-F442A induction.

Clones A and B efficiently commit multipotential C3H10T1/2 and precrisis mouse and rat fibroblasts to adipogenesis

The 3T3-C2 cell line was originally selected for its inability to undergo adipogenesis upon confluence and insulin induction (3, 4). Because 3T3-C2 and the preadipocyte line 3T3-F442A had a common origin, we were concerned that Clones A and B might be complementing a mutation in 3T3-C2 cells downstream from the commitment step. The C3H10T1/2 (10T1/2) line is multipotential; when treated with 5-azacytidine, determined myogenic, adipogenic, or chondrogenic cell lineages can be derived at low frequencies (13, 14). Clone A or B DNA co-transfected with pKoNeo into 10T1/2 cells is each equally able to commit those cells to efficient rapid adipogenesis in the absence of 5-azacytidine (Table 1). *Hind*III digestion, which reduces the AC activity of p18A4 cosmid, also drastically decreases the frequency of adipocyte conversion in 10T1/2 cells transfected with restricted Clone A DNA (data not shown). The conversion of control or pKoNeo-transfected 10T1/2 cells to adipocytes was negligible.

Clone A can commit precrisis mouse and rat fibroblasts to adipogenesis. The ability of Clone A to induce fat conversion in precrisis mouse cells was tested using C57L primary mouse skin fibroblasts (MSF) (15). Eight of twenty-three Neo^r MSF transfectant clones isolated were positive for adipocyte differentiation (Table 1). The background of spontaneous fat-cell conversion exhibited by MSF cells is low (Table 1). MSF clones

Table 2. Clonal commitment of pre-crisis mouse skin fibroblasts by Clone A DNA.

Subclone	First foci observed (week)	Range of fat foci/plate	
		Isolated	Thawed
MSFA-1	1	$>10^3$	$>10^3$
MSFA-2	1	$>10^3$	78–81
MSFA-3	–	0	0
MSFA-4	–	0	0
MSFA-5	1	10–15	9–15
MSFA-6	1	37–77	$>10^3$
MSFA-7	1	39–65	$>10^3$

Clone A DNA was co-transfected with neomycin-resistance plasmid pKoNeo into C57L MSF cells. Individual G418^r colonies were expanded and split into sister cultures. One culture was frozen and the other was expanded into three dishes and assayed for AC. Once MSF clones were found to be positive or negative, a frozen sister culture was thawed and retested.

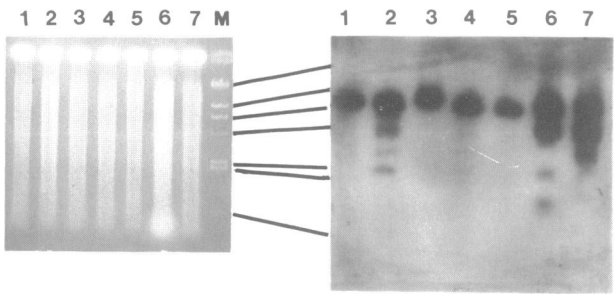

Figure 1. Southern blot hybridization of Clone A DNA in MSFA cells. *Pst*I-restricted genomic DNA from seven MSFA cell lines was electrophoresed through a 0.8% agarose gel, transferred to Genescreen Plus, and probed with ^{32}-P-labeled, PCR-amplified Clone A insert. Lane 1; parental cells of MSFA, lanes 2 through 7 are DNAs from MSFA-5, MSFA-6, MSFA-3, MSFA-4, MSFA-1 and MSFA-7 respectively. M= Lambda *Hind*III molecular weight markers (from the top): 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0 kb. Note presence of additional integrated Clone A DNA bands in MSFA-5, MSFA-6, MSFA-1 and MSFA-7 cell lines: these lines converted to adipocytes upon induction with insulin at confluence; the other MSFA clones lack exogenous Clone A bands and do not convert.

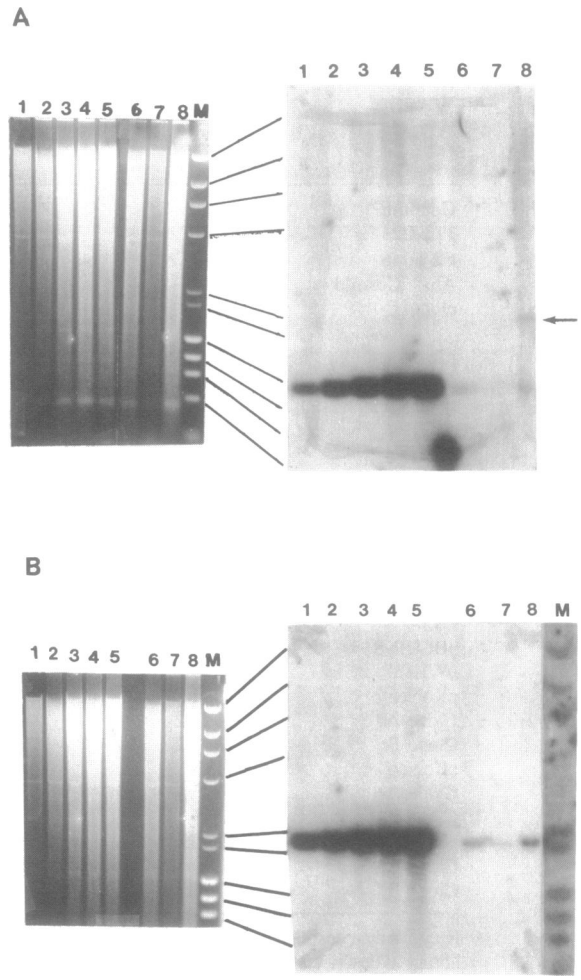

Figure 2. Southern blot hybridization of Clones A and B to *Bam*HI-restricted mouse genomic DNAs. 5–10 μg *Bam*H1-restricted DNA from cultured mouse cells were electrophoresed through a 0.8% Agarose gel, transferred to Immobilon-N membrane (Millipore) and hybridized overnight to ^{32}P-labeled Clone A or B gel-purified insert. **A.** DNAs hybridized to 1.2 kb (Clone A) probe; **B.** DNAs hybridized to 2.0 kb (Clone B) probe. Lanes 1–8: 9A, 3T3-F442A (induced), 3T3-F442A (uninduced), 3T3-C2, Swiss 3T3; 10T1/2, C57L MSF, and NIH-3T3 DNAs respectively, M= Lambda *Hind*III φX174 *Hae*III molecular weight markers (from the top): 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 1.4, 1.1, 0.9 and 0.6 kb. Note the high copy number of sequences hybridizing to Clones A and B in subclones of Swiss 3T3 cells, including the secondary transfectant 9A from which the library was derived. On longer exposures a second fragment, 2.3 kb in size (arrow), can be detected hybridizing to Clone A in Swiss 3T3 and NIH 3T3 DNAs. We do not know the significance of this second band in terms of adipose commitment.

that most completely responded to confluence and insulin induction contained an uncountable number ($>10^3$) of fat foci per dish (Table 1 & 2). Several frozen Clone A-transfected MSF clones (MSFA series) were thawed out, grown, and tested for adipocyte conversion. Upon thawing and subculture, negative clones remained negative. There was some variability in initially positive clones; some showed an increase in the number of fat foci upon thawing and retesting (MSFA-6, MSFA-7), while others showed a reduction (MSFA-2), and still others remained the same (MSFA-1, MSFA-5). RNA was isolated from induced parental MSF and two induced transfected clones, MSFA-1 and MSFA-3. Northern blots demonstrated expression of aP2 mRNA, the adipocyte-specific, myelin-P2 like fatty-acid binding protein, and glycerophosphate dehydrogenase in the positive MSFA-1, whereas the parent MSF and the negative MSFA-3 had no detectable levels of these mRNAs (data not shown). There is little difference between the MSFA high responder lines and 3T3-F442A in the time courses of their differentiation in culture and number of fat foci per dish.

Clones A and B also induce fat conversion in precrisis rat cells. F2408 rat embryo fibroblasts (6) were transfected with Clone A or B. Fat cells were observed in 14/24 plates transfected with Clone A and 19/24 plates with Clone B (Table 1). Clones A and B were both active above the low background of spontaneous conversion of rat embryo cells (Table 1).

Clones A and B are integrated in clonal lines committed to adipogenesis, but not in those which remain uncommitted after transfection

DNAs were isolated from MSFA clonal lines that tested either positive or negative in our transfection assay. As the MSF transfectants were cloned from single Neo^r colonies, rather than pooled, an analysis of integrated DNA was facilitated. Southern analysis using the insert from Clone A as probe demonstrated an endogenous 6.5 kb band in untransfected *Pst*I-restricted MSF DNA (Figure 1). All positive MSFA clones showed a single-

copy intensity endogenous sequence, plus at least one copy of integrated Clone A DNA, while negative clones showed no integrated sequences (Figure 1). The same is true for precrisis MSF cells transfected with Clone B (MSFB series). Three MSFB clones have been tested: two are AC positive and have endogenous (1.2 and 5.0 kb *Pst*I fragments) plus integrated Clone B DNA, and one is negative and lacks an integrated sequence.

Endogenous mouse sequences hybridizing to clones A and B appear reiterated in Swiss 3T3 cells and their descendants

Analysis of genomic DNA showed that the endogenous mouse sequences hybridizing to Clone A or B are reiterated approximately 50 times in Swiss 3T3 cells and derivatives (Figure 2A for Clone A, and 2B for Clone B). The estimate is based on comparative measurements of signal intensity on Southern blots using the Molecular Dynamics 400A phosphorimager (16). In contrast, 10T1/2, C57L MSF and NIH-3T3 cells contain a low copy number of sequences hybridizing to Clones A and B (Figures 2A and 2B). Because Clones A and B had the same AC activity in 3T3-C2, 10T1/2 and C57L MSF cells, it is unlikely that the extra endogenous copies in 3T3-C2 cells are relevant to their response to Clones A and B.

Absence of clone A or B homology to MyoD1-like helix-loop-helix sequence

The MyoD gene family of mammalian skeletal muscle regulators, which include MyoD1 (17), Myf5 (18), myogenin (19), and herculin (20) induce myogenesis in a variety of cells. They belong to a larger family of DNA binding proteins which share a conserved stretch of amino acids: a basic amino acid region followed by a region predicted to have a helix-loop-helix (HLH) secondary structure; that is, two successive amphipathic helices separated by a loop (21). Only a 68 amino acid stretch of MyoD1 containing this region is needed for stable myogenic conversion of recipient cells (22). The highly conserved amino acid sequences of the HLH region of the mouse and human members of the MyoD family were used to design a 40 bp oligonucleotide that included 9 inosines, and is completely within one exon. This oligonucleotide was used as a probe to screen *Bam*H1 restriction fragments of p18A4, including Clones A and B inserts, and of three MyoD-family genes (myogenin, herculin, MyoD1) as positive controls. Under conditions of high stringency only the MyoD family plasmids hybridized to our HLH probe (data not shown). By sequence and Southern blot analysis we have shown that Clones A and B are not likely to encode an HLH protein with high homology to the MyoD family HLH. Another muscle regulating gene, *myd*, described by Pinney *et al.* (23), shows no homology to MyoD1. *Myd*, a myogenic commitment gene derived from a demethylated human genomic cosmid library, acts earlier than the MyoD1 gene (24). Because *myd* has not been extensively characterized nor its sequence obtained, homologies between it and Clone A or B cannot be determined at this time.

Clones A and B are novel gene sequences

Both strands of genomic Clones A and B have been sequenced. Genbank Accession Numbers L04495 (Clone A) and L04496 (Clone B). Sequences failed to show concordant patterns for splice sites, enhancer, promoter, CAAT, TATA, polyadenylation, and other recognition sequences, nor did a scan of all possible frames reveal a preferred open reading frame. The longest open reading frame of Clone A encodes about 60 amino acids and that of Clone B about 80 amino acids. No significant homologies to previously sequenced genes or domains have been found in searches of the Genbank and EMBL databases.

DISCUSSION

The small genomic Clones A and B are each capable of committing transfected mouse and rat cells to adipogenesis. This effect was not seen with any of the other eight DNA fragments subcloned from cosmid p18A4. Furthermore, the AC activity was reduced when *Hind*III-restricted cosmid p18A4 or Clone A or B was used as donor DNA. Both Clones A and B have internal *Hind*III sites. Clones A and B are functional in rodent cells, both clones efficiently committing pre-crisis and multipotential fibroblast cells to adipocytes. Adipocytes obtained by transfection of fibroblasts with Clones A or B do not differ phenotypically from 3T3-F442A cells. When one considers the percentage of drug-resistance plates showing fat foci, no increase is noted when comparing genomic *vs.* cloned DNA as donor (Table 1, last column). As the active sequences were purified from genomic, to cosmid, to pUC-subcloned AC DNA, an increase in specific activity was indicated by the dramatic increase in the number of fat foci [genomic C2/F046 (2.8) *vs.* cosmid clone p18A4 (12.7) *vs.* Clone A (144.5) or Clone B (164.5)] and the shortening of the time course for the foci to appear (5 weeks *vs.* 3 weeks *vs.* 1 week).

The mechanism of action of Clones A and B remains to be elucidated. In Southern blots, Clones A and B fail to hybridize to each other, or to the inserts of the other 4 active cosmids derived from the same library (data not shown). This indicates there are 3 or more different AC active sequences which we have cloned. The nucleotide sequences of Clones A and B have not been previously reported and contain no prominent structural motif. Nevertheless, despite their small sizes, Clones A and B are acting functionally as genes in our assay. The most likely mechanism would involve as a first step the transcription of a Clone A- or B-encoded RNA product. The vector we used does not provide enhancers, promoters, splice or polyadenylation signals; therefore, should either Clone A or B code for a protein, it would have to include at least a minimal operative regulatory region as well. Northern blots with RNA isolated one day post-confluence failed to detect any differential RNA product hybridizing to Clones A or B; similarly, no positive signal was seen in a screen of a human fat tissue cDNA library (data not shown). However, these data are very limited and can only be considered preliminary, since a lack of demonstrable RNA product may be indicative of its low abundance or perhaps its temporal regulation. If Clone A or B is a commitment gene, it need not be transcribed continuously or at high levels. Experiments are underway to examine RNAs at all time periods from growth through induction using the more sensitive RNase protection assay.

Clones A and B were isolated from a cosmid library of 9A, a pool of committed preadipocyte cells obtained from a secondary transfectant clone containing donor human fat tissue DNA. Donor DNAs from 3T3-C2 cells or from 3T3-C2/3T3-C2 secondary transfectants have never produced fat foci in our AC assay ((5) and Table 1). Therefore, the AC activity of Clones A and B must be a consequence of the original human DNA transfection that gave rise to clone 9A. However, Southern blot analysis of various human genomic DNAs hybridized with Clone A and B inserts

failed to show consistent hybridization under stringent conditions. The puzzling observation leaves open the possibility that recombinations occurred during the primary or secondary transfections, or cosmid cloning. In the case of MSF cells, a single integrated copy of either Clone A or Clone B DNA is sufficient for commitment to adipogenesis (Figure 1), so endogenous and integrated sequences must differ in ways that are germane to the commitment process. The possibility that Clones A or B contain a DNA binding site for a regulatory protein and do not encode for either protein or RNA has not been ruled out.

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