Regulation of the Human hsp70 Promoter by p53

S. Nicholas Agoff, Jeannie Hou, Daniel I. H. Linzer, Barbara Wu*

The tumor suppressor p53 is a nuclear phosphoprotein with characteristics of a transcription factor. It displays sequence-specific DNA binding, contains a potent transactivation domain, and has been implicated as both a transcriptional activator and a repressor. Transcription of the human *hsp70* gene is stimulated by adenovirus E1a protein. This E1a transactivation of the hsp70 promoter is mediated by CCAAT binding factor (CBF). It is demonstrated here that p53 both represses transcription from the human hsp70 promoter and also interacts with CBF. Thus, the repression of the hsp70 promoter by p53 may be mediated by direct protein-protein interaction with CBF. These results suggest that protein-protein interaction between p53 and specific transcription factors may be an additional mechanism by which p53 regulates gene expression.

The tumor suppressor p53 is a nuclear phosphoprotein involved in the control of cell proliferation, and mutations in the *p53* gene are commonly found to be associated with diverse types of human cancer (*1, 2*). The antiproliferative effect of wild-type p53 is demonstrated by its ability, when overexpressed, to inhibit oncogene-mediated cellular transformation (*3, 4*). A common target of transforming proteins of DNA tumor viruses, p53 associates with SV40 T-antigen (*5*), adenovirus E1b (*6, 7*), and human papilloma virus E6 (*8*). These associations are thought to repress the activity of wild-type p53 by targeting it for rapid degradation (*9*) or by sequestering it in inactive complexes (*10*).

Wild-type p53 displays two activities characteristic of a transcription factor: DNA binding (*11*) and transcriptional activation (*12, 13*). Thus, p53 may function by regulating the transcription of genes whose products suppress cell proliferation. Although these putative targets have yet to be identified, p53 does repress the transcription of several growth-regulated genes (*14*), consistent with a role for p53 in growth arrest at the G_1-S boundary of the cell cycle (*15*). Loss of wild-type p53 function might therefore transcriptionally activate those genes normally repressed by p53. This pattern of repression by wild-type p53 and activation by mutant p53 has been observed for the promoter of the multidrug-resistance gene (*16*).

We examined the effects of wild-type and mutant p53 on the growth-regulated human *hsp70* gene. The *hsp70* gene is induced by serum (*17*) and by adenovirus E1a (*18*) and is expressed in the late G_1 early S phase of the cell cycle (*19*), an expression pattern similar to that of p53 (*20*). One element necessary for the growth-regulated and E1a-induced expression of the *hsp70* gene is a CCAAT element at promoter position −70. This element is recognized by a specific CCAAT binding factor (CBF), which stimulates transcription from the hsp70 promoter (*21*) and mediates E1a transactivation of the hsp70 promoter through a physical interaction between CBF and E1a (*22*). This productive interaction with viral E1a suggests that the activity of CBF may normally be regulated by an association with a cellular factor. We report here that p53 binds to CBF and regulates the activity of this transcription factor on the hsp70 promoter. Thus, CBF represents the first transcription factor identified as a target of p53 action.

Cotransfection of the hsp70 promoter–CAT reporter with an expression construct encoding wild-type human p53 into Chinese hamster (CHO) cells reduced transcription from the hsp70 promoter. By comparison, mutant forms of p53, such as *175his* and *273his* (*2*), which are associated with many human tumors, displayed less repression or no repression (Fig. 1A). Unlike the response of the hsp70 promoter, the α-globin promoter, which also contains an essential CCAAT element (*23*) but is not responsive to CBF (*21*), is not affected by p53 (*24*). The responsiveness of the hsp70 promoter, but not the α-globin promoter, to CBF and p53 suggests a possible role for p53 in the regulation of CBF function. This notion was supported by the ability of p53 to repress CBF-stimulated transcription from the hsp70 promoter in COS cells (Fig. 1B). Lum *et al.* have dem-

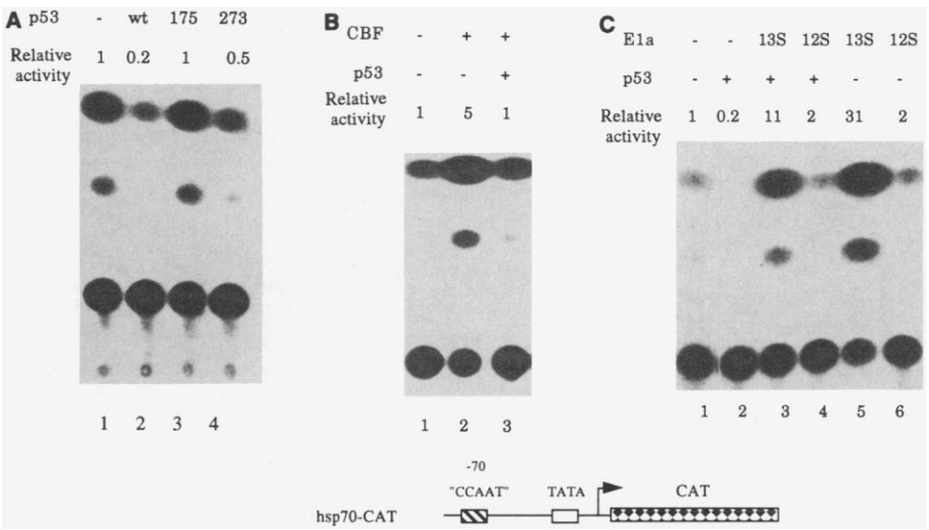

Fig. 1. Effects of p53 on transcription from the hsp70 promoter. Enzymatic chloramphenicol acetyltransferase assays conducted (*33*) and quantified by phosphorimage analysis. (**A**) Wild-type p53 represses transcription to a greater degree than mutant forms of p53. CHO cells cotransfected by DEAE-dextran procedure (*34*) with hsp70 promoter–CAT reporter gene and pC53-C1N_3, pC53-4.2N_3, or pC53-Cx22AN_3, encoding wild-type (wt) or mutant (*273his* or *175his*) forms of human p53, or vector only (−). Transfections were repeated four times, relative activities were calculated (based on lane 1 = 1.0) for each experiment, and means and standard deviations were derived: lane 2, 0.2 ± 0.08; lane 3, 1.0 ± 0.12; lane 4, 0.5 ± 0.17. (**B**) Wild-type p53 represses CBF-induced transcription from hsp70 promoter. COS cells were cotransfected by the DEAE-dextran procedure (*34*) with hsp70-CAT reporter gene and expression constructs pMt-CBF or pC53-C1N_3, encoding the indicated polypeptides (+), or vectors only (−). Transfections were repeated two times, relative activities were calculated, and means and standard deviations were derived: lane 2, 7 ± 2.8; lane 3, 1.5 ± 0.17. (**C**) Adenovirus E1a relieves repression caused by p53. CHO cells were cotransfected with hsp70-CAT reporter gene and expression constructs pJN20, pJN12, or MSVcL, encoding adenovirus E1a, 13*S* or 12*S*, or murine p53 (+), respectively, or vector only (−). Transfections were repeated three times, relative activities were calculated, and means and standard deviations were derived: lane 2, 0.2 ± 0.0; lane 3, 10.5 ± 0.7; lane 4, 1.9 ± 0.14; lane 5, 37.5 ± 9.2; lane 6, 3.3 ± 1.9. Plasmid sources are as referenced (*35*).

Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60208.

*To whom correspondence should be addressed.

onstrated that transfected CBF promotes transcription from the hsp70 promoter in COS cells in a promoter-selective and CCAAT box–dependent manner (*21*). The repression is specific to wild-type p53 and is not the result of decreased expression of transfected CBF as assessed by immunoprecipitations of pulse-labeled proteins (*24*).

In addition, two observations suggest that viral E1a and host-cell p53 might regulate *hsp70* gene expression through the same regulatory pathway: (i) p53 can revert E1a-mediated cellular transformation (*3*) and (ii) E1a and CBF can functionally interact (*22*). If both E1a and p53 regulate the hsp70 promoter through CBF, one might expect that E1a would relieve the repression caused by p53 and that p53 would limit the extent of E1a transactivation. The repression of the hsp70 promoter in CHO cells caused by p53 can be relieved by either the 13S or 12S form of E1a, and the 31-fold stimulation attained by E1a 13S alone in CHO cells was reduced 60% in the presence of p53 (Fig. 1C). Furthermore, this effect was titratable; increasing amounts of p53 reduced the level of E1a transactivation and increasing amounts of E1a reduced the extent of p53 repression (*24*). In contrast, the twofold stimulation attained by E1a 12S was unaffected by p53. In terms of *hsp70* gene expression, E1a 13S differs from 12S in its ability to interact with CBF in vivo and in vitro (*22*), suggesting that p53 repression of E1a 13S transactivation may involve alterations in CBF protein complexes.

Altered CBF complexes might arise if p53, like E1a, is able to bind to CBF. To test for an association of CBF and p53 in vivo, we took advantage of the observation that both GAL-E1a (*25*) and GAL–murine p53 (*13*) fusion proteins display potent transcriptional activation of G5CAT, a synthetic reporter gene containing five GAL4 binding sites (*25*). Unlike p53 and E1a, GAL-CBF does not exhibit such transactivation capability in CHO cells in the absence of cotransfected E1a (*22*). If p53 and CBF interact, an intermolecular GAL-CBF:p53 complex should transcriptionally activate G5CAT in a manner analogous to that observed for E1a 13S and GAL-CBF (*22*). As shown in Fig. 2A, GAL-CBF mediated transactivation of G5CAT by p53 and by E1a, but not by either creb2 or c-myc. This effect was dependent on the CBF(1-192) peptide moiety because transcriptional activation was not observed with other GAL-CBF fusions containing deletions of the NH_2-terminus of CBF (*24*). This effect was also dependent on the transactivation function of p53. Mutant *175his* was defective for transactivation as a GAL fusion protein (*2*) and, similarly, this mutant form of p53 was unable to promote transcription directed by GAL-CBF (Fig. 2B, lane 3). The absence of induction in this case may also be due to the failure of *175his* to interact with CBF. In contrast, mutant *273his* transactivated as a GAL fusion protein (*12*) and also promoted transcription directed by GAL-CBF (Fig. 2B, lane 2). These genetic data suggest that p53 and CBF interact in vivo.

To verify that these proteins can physically associate, we assayed the ability of wild-type p53 to bind to CBF in vitro using two methods: affinity chromatography and co-immunoprecipitation. The region of CBF sufficient to mediate E1a (*22*) and p53 (Fig. 2B) induction of G5CAT was contained within CBF residues 1 to 192. Therefore, bacterially expressed glutathione *S*-transferase (GST)–CBF(1-192) fusion protein was bound to glutathione agarose and used as an affinity resin. In vitro translated p53 was retained by GST–CBF(1-192), but not by GST alone (*24*) or by other fusion proteins, such as GST-topoisomerase (GST-Topo) (Fig. 3A, lanes 2 and 3). Conversely, polypeptides unrelated to p53, such as Hsp70, were not retained by GST-CBF (Fig. 3A, lanes 5 and 6).

Co-immunoprecipitation of in vitro translated polypeptides also detected an association of p53 and CBF (Fig. 3B, lane 3). When coincubated with unlabeled CBF, labeled p53 was recovered in an anti-CBF immune complex. As expected, E1a was also coimmunoprecipitated in the presence of CBF but Hsp70 was not recovered in this assay (Fig. 3B, lanes 1 and 2, respectively).

If p53 and CBF interact in vivo, we would expect to detect CBF in an anti-p53 immune complex from cell lysates, whereas we would not expect to find Hsp70 in these complexes. We were able to attain equivalent expression of CBF and endogenous Hsp70 (Fig. 3C, lanes 2 and 3) in CBF-transfected COS cells. Immune complexes recovered from these cells by anti-p53 were released and assayed for the presence of CBF or Hsp70 by immunoprecipitations with anti-CBF or anti-Hsp70, respectively. These double immunoprecipitations detected an in vivo association of CBF, but not Hsp70, with p53 (Fig. 3C, lanes 5 and 6).

Protein-protein interactions may be required for p53 regulation of the cell cycle. A physical interaction of p53 with SV40 T-antigen limits the ability of T-antigen to associate with DNA polymerase α and to promote SV40 DNA replication (*26*). An analogous association may occur between p53 and a cellular factor essential for DNA synthesis, thus preventing the progression of cells into the S phase. In contrast, the

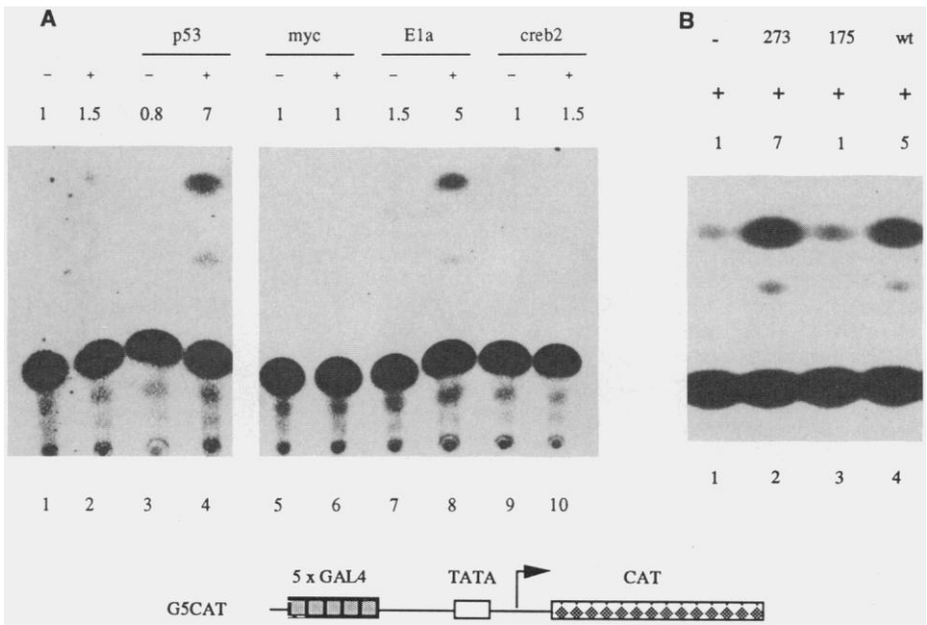

Fig. 2. Genetic evidence for in vivo interaction between p53 and the NH_2-terminal 1 to 192 residues of CBF. (**A**) Wild-type p53 stimulates transcription directed by GAL–CBF(1-192) fusion protein. CHO cells were co-transfected with the G5CAT reporter gene in the presence (+) or absence (−) of pGAL-CSSP, encoding GAL–CBF(1-192), along with expression vectors MSVcL, pSVcmyc1, pCE, or pJ8-creb2, encoding murine p53, c-cmyc, E1a, or creb2, respectively. (**B**) The *273his* mutant form of p53, but not the *175his* form, also stimulates transcription directed by GAL–CBF(1-192). CHO cells were cotransfected with G5CAT reporter gene pGAL-CSSP (*35*) and expression vectors MSVcL, pC53-4.2N_3, or pC53-Cx22AN_3, encoding the indicated forms of p53. Transfections were repeated four times, and means and standard deviations were derived: lane 2, 6 ± 1.4; lane 3, 1 ± 0.0; lane 4, 5.5 ± 0.7. Plasmid sources are as referenced (*35*).

effect of p53 on transcription has been thought to involve direct contact of p53 with a specific DNA sequence (*11*), resulting in either the repression of growth-regulated genes (*14*) or the induction of genes expressed upon terminal differentiation, such as muscle creatine kinase (*27*). The results with the hsp70 promoter indicate that p53 may also utilize protein-protein interaction with specific transcription factors to regulate transcription.

We are confronted with the paradoxical observation that—although both E1a and p53 function as strong transcriptional activators—for the hsp70 promoter, CBF complexed with E1a is an activator, whereas CBF complexed with p53 is a repressor. Perhaps a p53-CBF complex alters the ability of CBF to recognize the hsp70 promoter. Functional antagonism between two positively acting transcription factors resulting from protein-protein interaction has been documented for glucocorticoid receptor and AP-1 (*28*), as well as for myoD and c-jun (*29*). Alternatively, CBF may inhibit the transactivation function of p53. The ability to modulate this function has been demonstrated for mutant p53 (*27*), adenovirus E1b (*7*), and mdm2 (*30*); each of these inhibits p53-mediated transactivation of the muscle creatine kinase promoter. Our data on the transcriptional competency of GAL-CBF complexed to p53 suggest that CBF does not interfere with the transactivation function of p53. Of course, this synthetic configuration may not accurately reflect the phenotype of native CBF and p53 on their natural targets.

In response to growth stimuli, wild-type p53 undergoes a conformational change during the G_1 stage and adopts a mutant-like conformation (*31*), and some mutant forms of p53 can oligomerize with wild-type p53 and alter its conformation (*32*). Therefore, mutant forms of p53 may alter the wild-type p53-CBF complex, perhaps by promoting the dissociation of wild-type p53 from CBF, even in the absence of growth stimuli. The ability of p53 to associate with CBF and to regulate CBF activity suggests that p53 might function by protein-protein interactions to control transcription from promoters without p53 binding sites. Thus, additional CBF-dependent genes, as yet unidentified, may be regulated by p53, and other transcription factors may interact with p53.

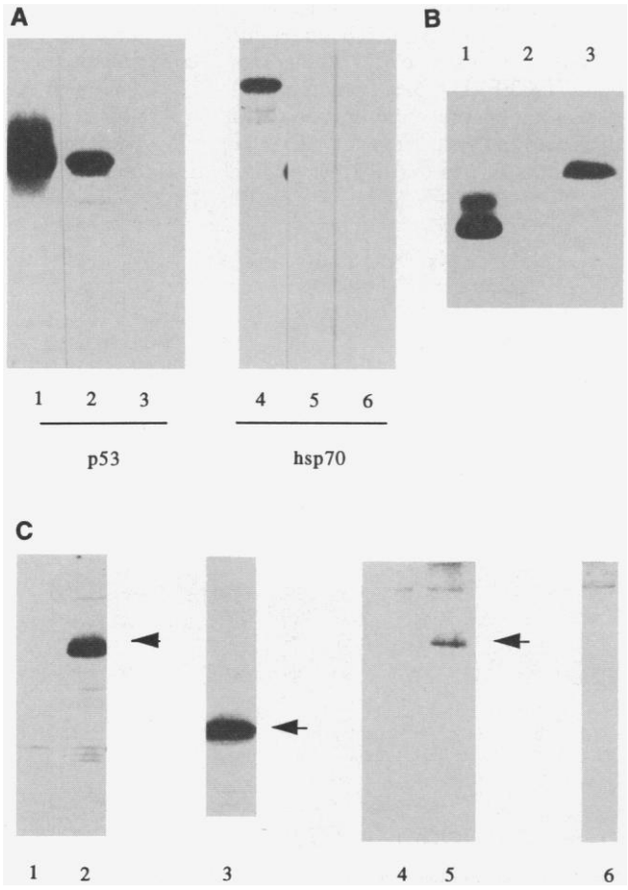

Fig. 3. (**A**) In vitro interaction between p53 and the NH_2-terminal 1 to 192 residues of CBF. Equal amounts of various GST fusion proteins synthesized in *Escherichia coli* and bound to glutathione-Sepharose were generated as described (*21*). In vitro transcribed and translated ^{35}S-methionine–labeled p53 or Hsp70 was directly applied to SDS-PAGE (polyacrylamide gel electrophoresis) (lanes 1 and 4) or fractionated as previously described (*22*) on beads affixed with GST–CBF(1-192) (lanes 2 and 5) or GST-Topo (lanes 3 and 6). The bound fraction was subjected to SDS-PAGE. (**B**) Coimmunoprecipitation of p53 and CBF. Equal trichloroacetic acid (TCA) precipitable counts of in vitro translated and labeled polypeptides E1a, hsp70, and p53 (lanes 1 through 3), respectively, were coincubated with in vitro translated and unlabeled CBF and subsequently immunoprecipitated with anti-CBF mAbO34. The immune complexes were subjected to SDS-PAGE. (**C**) Double immunoprecipitation detects a complex of CBF and p53 in vivo. Lysates prepared from transfected COS cells labeled with ^{35}S-methionine were immunoprecipitated with anti-CBF mAbO34 (lanes 1, vector only; lane 2, CBF) or with anti-Hsp70 (C92, Amersham; 3A3, Affinity Bioreagents; lane 3, endogenous Hsp70). For double immunoprecipitations (lanes 4 through 6), lysates used in lanes 1 through 3 were first immunoprecipitated under native conditions (*7*) with anti-p53 PAb421 (*36*). Polypeptides recovered in these immune complexes were released by boiling in Laemmli buffer, diluted, and subjected to a second immunoprecipitation essentially as previously described (*36*) with anti-CBF (lanes 4 and 5) or anti-Hsp70 (lane 6). Plasmid sources are as referenced (*35*).

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Adipose Expression of Tumor Necrosis Factor–α: Direct Role in Obesity-Linked Insulin Resistance

Gökhan S. Hotamisligil, Narinder S. Shargill, Bruce M. Spiegelman*

Tumor necrosis factor–α (TNF-α) has been shown to have certain catabolic effects on fat cells and whole animals. An induction of TNF-α messenger RNA expression was observed in adipose tissue from four different rodent models of obesity and diabetes. TNF-α protein was also elevated locally and systemically. Neutralization of TNF-α in obese *fa/fa* rats caused a significant increase in the peripheral uptake of glucose in response to insulin. These results indicate a role for TNF-α in obesity and particularly in the insulin resistance and diabetes that often accompany obesity.

Obesity and diabetes are among the most common human health problems in industrialized societies. Obesity, which is the result of an imbalance between caloric intake and energy expenditure, is highly correlated with insulin resistance and diabetes in experimental animals and humans. However, the molecular mechanisms that are involved in obesity-diabetes syndromes are still not clear. Because adipose tissue is the major site for energy storage and mobilization, many studies have been focused on finding abnormalities in adipocyte physiology or metabolism (*1*, *2*).

Several cytokines, such as TNF-α, have important metabolic effects (*3*, *4*), including direct effects on adipocyte metabolism. TNF-α acts in vitro on murine adipocytes to suppress expression of most adipose-specific genes, including the enzymes involved in lipogenesis (*5*, *6*). However, some of these effects are not observed in primary cultures of human or rat adipocytes (*4*, *7*). In vivo, TNF-α expression has been associated with catabolic states leading to a wasting syndrome, termed cachexia (*8*, *9*), but this effect of TNF-α has been challenged by several groups of investigators (*10*, *11*). TNF-α administration causes an increase in serum triglycerides and very low density lipoproteins in rats and humans (*10*, *12*). This hyperlipidemia is thought to be the result of decreased lipoprotein lipase activity and increased hepatic lipogenesis (*13*). TNF-α administration also has effects on appetite and gastrointestinal tract functions (*1*). Besides TNF-α, other cytokines, such as TNF-β, interleukin-1 (IL-1), IL-6, and interferon, also have profound effects on lipid metabolism (*4*). Furthermore, all of these cytokines affect glucose homeostasis in various tissues (*14*).

Our laboratory has been pursuing the role of cytokines in energy balance and fat metabolism, primarily because of their potential role as endogenous regulators of gene expression in fat tissue. We have shown that fat cells produce certain key components of the alternative pathway of complement, including adipsin–factor D (*15–17*). The local complement pathway appears to be dysregulated in several models of obesity and can be controlled in cultured adipocytes by certain cytokines, including TNF-α (*16*, *17*). This link between the immune system and energy metabolism has led us to investigate directly the regulation and role of cytokines in obesity-diabetes syndromes.

To examine the expression of the TNF-α gene in the tissues of lean (+/?) or obese (*db/db*) mice, we extracted total RNA from various tissues and organs and subjected them to RNA (Northern blot) analysis (Fig. 1). Endogenous expression was evident only in adipose tissue and spleen. The amount of TNF-α mRNA expression in spleen was not different in obese mice as compared with their lean litter mates. However, in adipose tissue the amount of TNF-α mRNA per unit of RNA was at least five- to tenfold elevated in obese animals, as compared with lean controls. TNF-β, IL-1α, IL-1β, and IL-6 were neither expressed in fat tissue nor regulated in any other organ in obesity (*18*). The earliest time of adipose expression of TNF-α examined was 6 to 7 weeks of age in *db/db* mice and 3 to 4 weeks of age in *fa/fa* rats (*18*), when animals are obese and insulin-resistant but not significantly hyperglycemic (*19*). TNF-α mRNA in fat tissue was elevated at these times.

Besides adipocytes, adipose tissue consists of vascular endothelial cells, smooth muscle cells, fibroblasts, local mast cells, and macrophages (*20*). To determine the source of TNF-α expression in adipose tissue, we separated mature adipocytes and nonadipose cells (stromal-vascular fraction), as described (*21*), and determined the amount of mRNA associated with these compartments. The majority of the TNF-α mRNA fractionated with the adipocytes, although some was also detected in the stromal-vascular fraction that contains nonadipocytes and less mature adipocytes

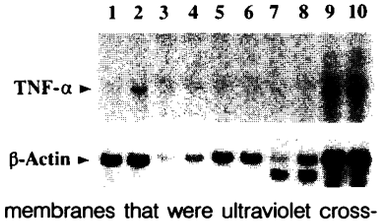

Fig. 1. Expression of TNF-α mRNA (indicated by the labeled arrow) in the tissues of lean and obese mice. Total RNA from tissues of 7- to 8-week-old, male, lean (+/?) and obese (*db/db*) animals (Jackson Laboratory) was extracted by a Cs chloride extraction protocol (*45*). Total RNA (20 μg) was denatured in formamide and formaldehyde at 55°C for 15 min and separated by electrophoresis in formaldehyde-containing agarose gels, as described (*44*). RNA was blotted onto Biotrans membranes that were ultraviolet cross-linked (Stratagene) and baked for 0.5 hours. Hybridization and washes were done as directed by the manufacturer. DNA probes were radioactively labeled to specific activities of at least 10^9 dpm/μg with [α-^{32}P]dCTP (6000 Ci/mmol) by the random priming method (*44*). Lanes 1 and 2, epididymal fat; lanes 3 and 4, liver; lanes 5 and 6, kidney; lanes 7 and 8, skeletal muscle; and lanes 9 and 10, spleen. Odd-numbered lanes, from lean animals; even-numbered lanes, from obese animals. β-Actin mRNA is shown as a control for the loading and integrity of the RNA. Lean mice are designated as +/? because +/+ and *db*/+ animals have not been differentiated.

Dana-Farber Cancer Institute and the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.

*To whom correspondence should be addressed at the Dana-Farber Cancer Institute.