Finkel-Biskis-Reilly Osteosarcoma Virus v-Fos Inhibits Adipogenesis and Both the Activity and Expression of CCAAT/Enhancer Binding Protein α, a Key Regulator of Adipocyte Differentiation

(Received for publication, August 5, 1997, and in revised form, October 8, 1997)

Derek W. Abbott† and Jeffrey T. Holt§
From the Departments of Cell Biology and Pathology and Cancer Center, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Finkel-Biskis-Reilly (FBR) osteosarcoma virus v-Fos causes tumors of mesenchymal origin, including osteosarcomas, rhabdomyosarcomas, chondrosarcomas, and liposarcomas. Because the cell of origin in all these tumors is a pluripotent mesenchymal cell, the variety of tumors seen in mice which express FBR v-Fos implies that FBR v-Fos inhibits multiple differentiation pathways. To study the mechanism of FBR v-Fos’ inhibition of mesenchymal differentiation, we utilized an in vitro model of adipocyte differentiation. We show by both morphological and biochemical means that FBR v-Fos inhibits adipocyte differentiation in vitro. This inhibition is due to FBR v-Fos’ inhibition of the growth arrest characteristic of terminal differentiation and FBR v-Fos’ inhibition of the expression and activity of a key regulator of this growth arrest, C/EBPα. The in vitro inhibition of adipogenesis by FBR v-Fos has in vivo significance as immunostaining of FBR v-Fos-induced tumors shows no CCAAT/enhancer binding protein (EBP)-α expression. These data implicate C/EBPα as a protein involved in the generation of liposarcomas.

Cellular differentiation and cancer are tightly linked. Clinically, cancers are graded according to their degree of differentiation. A high degree of differentiation of a cancer indicates a favorable prognosis while a low degree of differentiation indicates a poor prognosis (1). Despite the well established clinical relationship between differentiation and cancer, the molecular mechanisms by which this lack of differentiation occurs in high grade cancers are unknown. A model system to study this phenomenon has emerged through the use of the retroviral oncogene FBR v-fos.† FBR v-Fos is a fusion protein containing a retroviral gag gene, the mouse c-fos proto-oncogene, and the mouse c-fox gene (Fig. 1) (2–4). The retroviral gag sequence contains a myristylation site which alters FBR v-Fos’ carcinogenic potential (5). When FBR v-Fos is myristylated, transgenic mice which express this oncogene develop multiple uni-
differentiated tumors of mesenchymal origin, including rhabdomyosarcomas, chondrosarcomas, osteosarcomas, and liposarcomas (5). When the myristylation site of FBR v-Fos is altered in such a way that myristylation cannot occur (G2A v-Fos; Fig. 1), mice expressing this mutant retroviral oncogene develop only lipomas (5). The studies in the transgenic mice indicate that in vivo, FBR v-Fos can disrupt normal cellular differentiation pathways, and that since the effect on adipocytes is independent of the myristylation state of v-Fos, adipogenesis is particularly sensitive to inhibition by the Fos proteins.

FBR v-Fos is thought to cause carcinogenesis via a two-step mechanism (6). First, transformation must occur. Second, genomic instability must be created. Our laboratory has recently shown that while FBR v-Fos and G2A v-Fos both cause transformation, only cells which express FBR v-Fos are highly susceptible to DNA damage caused by ionizing radiation. Cells which express G2A v-Fos show normal sensitivity to ionizing radiation (6). These data imply that the difference between benign tumors and malignant tumors lies in genomic instability. However, to reach the malignant phenotype, transformation must also occur. Because either FBR v-Fos or G2A v-Fos expression leads to tumor formation (either liposarcomas or lipomas, respectively) both FBR v-Fos and G2A v-Fos must be inhibiting adipocyte differentiation, and studying this step may give insight into the first step of FBR v-Fos-induced carcinogenesis.

The study of adipocyte development has been made possible through the use of a pre-adipocyte cell line (NIH 3T3-L1) (8–10). When exposed to isobutylmethylxanthine, dexamethasone, and insulin, confluent NIH 3T3-L1 cells begin to change their cytoskeletal shape and eventually become adipocytes (10, 11). Adipogenesis is initiated and maintained by the sequential expression of three tissue-specific transcription factors; C/EBPβ, PPARγ2, and C/EBPδ (12–15). PPARγ2 is thought to be the major transcription factor responsible for the initiation of adipogenesis (14) while C/EBPα is thought to be the major protein involved in the maintenance of adipogenesis (13, 15). In the differentiated adipocyte, C/EBPα is the factor responsible for the expression of many fat cell-specific genes (16) including PEPCK (17), and ap2 (18). Expression of C/EBPα has been shown to be both sufficient and necessary for adipocyte development as ectopic expression of C/EBPα will convert pre-adipocytes to adipocytes in the absence of hormonal stimulation (19, 20), and antisense inhibition of C/EBPα expression inhibits adipocyte development (21). Expression of C/EBPα also causes growth arrest (19, 21–23), a key characteristic of terminally differentiated cells.

C/EBPα’s maintenance of the characteristic growth arrest of terminally differentiated adipocytes is implicated in the formation of liposarcomas. CHOP, a member of the C/EBP family of proteins, is an antagonist of C/EBP-mediated transactivation.
ther implicates the C/EBP family of proteins as key factors in vitro. The normal levels of C/EBP proteins begin the adipocyte differentiation pathway as they express the mechanism of this inhibition of adipocyte development. In myxoid sarcomas or lipomas in transgenic mice (3), we sought to study the mechanism of this inhibition of adipocyte development in vivo. We show that pre-adipocytes which express FBR v-Fos or G2A v-Fos do not differentiate into adipocytes. These cells begin the adipocyte differentiation pathway as they express normal levels of C/EBP, but later stages of differentiation are faulty as these cells do not express C/EBP. These cells also do not undergo the growth arrest which is characteristic of terminally differentiated cells. We further show that expression of FBR v-Fos or G2A v-Fos causes decreased transcriptional activity of C/EBPβ but not C/EBPβ. Lastly, by utilizing tumors from the FBR v-fos transgenic mice, we show that the expression pattern of the C/EBP proteins in vivo matches that seen in vitro. Thus, the use of FBR v-Fos to induce liposarcomas further implicates the C/EBP family of proteins as key factors in the generation of liposarcomas.

MATERIALS AND METHODS

Cell Culture and Transfection—NIH 3T3-L1 cells and HeLa cells were obtained from the American Type Tissue Collection (ATCC). These cells were grown in 10% fetal bovine serum (Sigma), 2 mM l-glutamine, 1 mM sodium pyruvate, and 100 units/ml penicillin-streptomycin. NIH 3T3-L1 cells were transfected with the indicated amounts of CRP-CAT, FBR v-Fos, or G2A v-Fos stable cell lines were generated by transfecting NIH 3T3-L1 cells with empty vector, FBR v-Fos or G2A v-Fos and the neomycin resistance gene (neo) in a 9:1 ratio using calcium phosphate precipitation. These cells were then selected for 2 weeks in 1 mg/ml G418 (Sigma). Approximately 500–1000 colonies were pooled and shown to express FBR v-Fos or G2A v-Fos by reverse transcription-polymerase chain reaction (5). CRP-CAT cells, CRP-CAT + FBR v-Fos cells and CRP-CAT + G2A v-Fos cells were generated in a similar manner, using a 9:9:1 ratio of CRP-CAT empty vector:neo, FBR v-Fos or G2A v-Fos:CRP-CAT:neo. HeLa cells were used for transient transfection assays. These cells were transfected with the indicated amounts of CRP-CAT, FBR v-Fos, G2A v-Fos, C/EBPα, or C/EBPβ and β-galactosidase. Cells were transfected with a total of 23 μg of DNA, using Pgem 7Zi (+) to standardize to equal amounts of DNA. Cells were transfected as described previously (30).

Plasmids Used—MSV-C/EBPα, MSV-C/EBPβ, FBR v-Fos, G2A v-Fos, and β-galactosidase were used as described previously (5, 31–33). CRP-CAT was obtained from Christian Trautwein and Michael Manns (Medizinische Hochschule Hannover) (34).

Differentiation Protocol—Initiation and maintenance of differentiation was performed as described previously (35). All experiments were performed on low passage-number cells. Briefly, at confluence (day 0 of differentiation), cells were fed with medium containing 10% fetal bovine serum, 0.5 mM isobutylmethylxanthine (Sigma), 10 μg/mL insulin (Sigma), and 1 μg dexamethasone (Sigma). Two days later, the medium was replaced with medium containing 2.5 μg/mL insulin and 10% fetal bovine serum. The cells were refed with this medium every 2 days for the remainder of the differentiation period. Cells were fixed in 10% formaldehyde, and Oil Red O staining was performed as described previously (35).

l-Glycerol-3-Phosphate Dehydrogenase (G3PDH) Assays—Cells were transfected with the indicated amounts of CRP-CAT and CRP-CAT stable cell lines, cells were differentiated for the time periods indicated. Cells were harvested and lysed by four cycles of freezing and thawing. Protein concentrations were standardized. CAT activity was assayed as described previously (31). For CAT assays on the transiently transfected HeLa cells, cells were harvested 48 h after transfection. Cells were lysed by four cycles of freezing and thawing. Transfection efficiency was then standardized using β-galactosidase activity by methods previously described (38).

Nuclear Protein Isolation and Electrophoretic Mobility Shift Assay (EMSAs)—R-SV-neo cells and FBR v-Fos cells were subjected to the differentiation protocol for 2 days. Nuclei were isolated and proteins extracted by the method described in Rana et al. (39). Protein concentrations were standardized, and EMSA was performed as described previously (39). Briefly, 10 μg of nuclear extract from the indicated cell line was incubated with 3 μg of poly(dI-dC) in 25 μl of buffer containing a final concentration of 150 mM KCl, 25 mM Hepes (pH 7.6), 7.5 mM glycerol, 0.1 mM EDTA, and 2.5 mM MgCl2. A double-stranded oligonucleotide probe containing the C/EBP binding site was replaced with 5′-GATCCGGCTT-GGCGCACAGTGC-3′ was end-labeled with α-32P]ATP. Protein binding to the oligonucleotide was determined by electrophoresis on a non-denaturing 6% polyacrylamide gel in TBE buffer (0.09 M Tris borate, 2 mM EDTA (pH 8.0)). In supershift studies, 5 μl of appropriate antibody were preincubated with the protein mixture for 1 h at room temperature prior to the addition of the probe.

Northern Blotting—After the days of differentiation, total RNA was harvested using the RNAeasy kit (Promega). Pgem7z-C/EBPα was digested with EcoRI/BamHI, and the resulting 1.4-kilobase fragment was isolated. This fragment was then random prime-labeled with [α-32P]dATP using the Prime-It II kit (Stratagene). 30 μg of RNA were electrophoresed on a formaldehyde gel and transferred to GeneScreen Plus. Prehybridization was performed with 200 μg/ml salmon sperm DNA, 6 x SSC, 50% formamide. Hybridization was then allowed to
and anti-C/EBP 
b proceed overnight. The blot was washed five times in 2
3
32456
zidine (DAB) chromagen using the following antibodies: anti-C/EBP
Rockville, MD) using a universal ABC detection kit with diaminoben-
ported (5). Processing of the tumor was conducted according to standard
FBR v-Fos. Generation of these transgenic mice has already been re-
ated preadipocyte, but as the cell begins to show overt signs of
approximately—
RESULTS
Both FBR v-Fos and G2A v-Fos Inhibit Adipocyte Development—The transgenic mouse model suggested that both FBR v-Fos and G2A v-Fos inhibited adipocyte differentiation (5). To study this phenomenon further, we utilized an in vitro model of adipocyte differentiation. NIH 3T3-L1 cells are a pre-adipocyte cell line which will differentiate into adipocytes upon exposure at confluence to insulin, isobutylmethylxanthine, and dexamethasone for 2 days followed by exposure to insulin for the remainder of the differentiation period. By day 8 of the differ-
entiation protocol, greater than 90% of the cells show overt
morphological signs of adipocyte differentiation (8–11). 3T3-L1
cell lines were made which stably expressed G2A v-Fos or FBR
v-Fos. These cell lines were shown to constitutively express
FRS-neo cell line. Both antibodies were diluted 1:100.

In addition to morphological study of these stably transfected
cell lines, we wanted to establish by biochemical means that
FBR v-Fos and G2A v-Fos cells do not differentiate into adipocytes. G3PDH is an enzyme whose activity increases greatly as a cell differentiates into an adipocyte (36, 37, 40). Measuring the biochemical activity of this enzyme is a well established method to assay adipocyte differentiation (41). After 8 days of differentiation, RSV-neo cells, FBR v-Fos cells, and G2A v-Fos cells were lysed, and biochemical activity of G3PDH was assayed. RSV-neo cells showed over 50-fold greater G3PDH ac-
tivity when compared with FBR v-Fos and G2A v-Fos cells (Table I), consistent with the fact that the RSV-neo cells dif-
ferentiate to adipocytes, while neither the FBR v-Fos cells nor
the G2A v-Fos cells differentiate to adipocytes.

To begin to determine the molecular mechanism for this inability to differentiate, growth curves of RSV-neo cells, FBR
v-Fos cells, and G2A v-Fos cells were measured. In normal
adipocyte differentiation, cells must be growth-arrested before
they began to show overt evidence of adipocyte differentiation (12, 42). Growth curves of differentiating pre-adipocytes should become relatively flat as the cells differentiate. To show that this is the case in our RSV-neo control cells, two plates of
RSV-neo cells were plated at equal cell density for each of the
six time points. At day 0, one plate of cells per time point began
the differentiation protocol, while the other plate of cells per
time point remained in normal media, maintaining a normal
growth rate. Cell counts were taken 0, 1, 2, 3, 5, and 8 days
after the beginning of the experiment. The RSV-neo cells re-
mained in normal medium showed exponential growth (Fig. 3A),
while those undergoing adipogenesis showed a signifi-
cantly lower rate of growth (Fig. 3B), consistent with differen-
tiation to adipocytes. Both FBR v-Fos cells and G2A v-Fos cells showed a growth curve similar to the RSV-neo cells in normal
media (Fig. 3A), showing that the expression of these oncogenes
does not increase the proliferation of these cells. Upon exposure
to differentiation media, however, the G2A v-Fos cells showed
no growth arrest, and the FBR v-Fos cells growth-arrested only
slightly (Fig. 3B). These results imply that FBR v-Fos and G2A
v-Fos do not inhibit adipocyte differentiation by stimulating
cell growth, but by inhibiting the growth arrest which is re-
quired for terminal adipocyte differentiation.

FBR v-Fos and G2A v-Fos Inhibit the Adipogenic Pathway
Downstream of C/EBPβ but Upstream of C/EBPα—The
C/EBP family of transcription factors regulates fat cell-specific
genes and is important for the initiation and maintenance of
adipocyte differentiation (15, 42). In an undifferentiated pre-
adipocyte, C/EBPβ is expressed at a low level. One day after
the initiation of the differentiation protocol, expression of
C/EBPβ is greatly induced before decreasing at later differen-
tiation stages (35). C/EBPα is not expressed in the undifferen-
tiated preadipocyte, but as the cell begins to show overt signs of

FIG. 2. FBR v-Fos and G2A v-Fos inhibit adipocyte differentiation. Stable pre-adipocyte cell lines were created which expressed either FBR v-Fos or G2A v-Fos (5). These cell lines were subjected to the differentiation protocol. After 8 days of differentiation, cells were fixed in formaldehyde and stained with Oil Red O (a stain of intracellular tryglycerides). Left panel, RSV-neo control cell line showing normal adipocyte differentiation. Middle panel, FBR v-Fos cell line showing no evidence of adipocyte differentiation. Right panel, G2A v-Fos cell line showing no evidence of adipocyte differentiation.

| Cell line | Specific activity ± S.E. |
|-----------|--------------------------|
| RSV-neo   | 1180 ± 9.40              |
| FBR v-Fos | 22.9 ± 7.20 a             |
| G2A v-Fos | 23.8 ± 16.1 a             |

a p values are <0.001 by Student’s t test when compared to the RSV-neo cell line.
The simplest explanation for this inhibition is that FBR v-Fos and G2A v-Fos cells implies that FBR v-Fos and G2A v-Fos inhibit this cycle. C/EBP expression pattern seen in FBR v-Fos and G2A v-Fos that these measurements have a \( p < 0.001 \) when compared with the RSV-neo cells (Student’s t-test).

adipocyte differentiation (days 3–5), C/EBP\( ^a \) begins to become expressed (35). Since both C/EBP\( ^b \) and C/EBP\( ^a \) are involved in a cascade regulation of adipocyte differentiation, we wanted to determine whether these proteins were expressed in FBR v-Fos and G2A v-Fos cells. By Western blot analysis, the RSV-neo cells show normal patterns of both C/EBP\( ^a \) and C/EBP\( ^b \) expression. C/EBP\( ^b \) is expressed early in differentiation, and expression lowers as the cell continues differentiating (Fig. 4, left/lower panel). In addition, the alternatively spliced C/EBP\( ^b \), liver inhibitory protein (43), which this antibody also recognizes, shows a similar expression pattern. C/EBP\( ^b \) begins to be expressed in the RSV-neo cells as the cell begins to reach the later stages of differentiation (Fig. 4, left/top panel). The expression pattern of these proteins is different in the FBR v-Fos and G2A v-Fos cells. In both cell lines, C/EBP\( ^b \) is expressed at levels indistinguishable from RSV-neo cells (Fig. 4, compare lower panels). Since C/EBP\( ^b \) expression is one of the earliest indicators of the initiation of adipogenesis (35), this implies that the early stages of adipogenesis are functioning normally in FBR v-Fos and G2A v-Fos cells. While the initiation of adipogenesis is unaltered, the expression pattern of C/EBP\( ^a \) shows that later stages of adipogenesis are faulty. Neither the FBR v-Fos cell line nor the G2A v-Fos cell line expresses C/EBP\( ^a \) (Fig. 4, upper middle and upper right panels; a positive control is shown in the far left lane of each blot). This expression pattern of C/EBP\( ^a \) is consistent with the fact that these cells do not differentiate. Moreover, the expression pattern of C/EBP\( ^b \) and C/EBP\( ^a \) suggests that FBR v-Fos and G2A v-Fos affect adipocyte differentiation between day 2 (normal expression of C/EBP\( ^b \) and days 3–5 (time when C/EBP\( ^a \) should begin to become expressed).

**FBR v-Fos and G2A v-Fos Inhibit Transcriptional Activity of C/EBP\( ^a \) but Not C/EBP\( ^b \)—C/EBP\( ^a \) transcriptionally regulates the expression of a number of adipocyte-specific genes (16) and is thought to be crucial for the maintenance of the adipocyte’s differentiated state (12, 42). Due to its effect on adipogenesis, C/EBP\( ^a \)’s expression is tightly regulated. Transactivation of the C/EBP\( ^a \) promoter by PPAR\( ^y \)/RXR and C/EBP\( ^b \) (44–48) allows C/EBP\( ^a \) to be expressed. Upon expression, C/EBP\( ^a \) regulates its own expression by binding to the C/EBP-binding site present in the C/EBP\( ^a \) promoter (44, 49). The C/EBP expression pattern seen in FBR v-Fos and G2A v-Fos cells implies that FBR v-Fos and G2A v-Fos inhibit this cycle. The simplest explanation for this inhibition is that FBR v-Fos and G2A v-Fos inhibit the transcriptional activity of C/EBP\( ^a \) and C/EBP\( ^b \). To test this hypothesis, a C/EBP-specific reporter construct (34, CRP-CAT; obtained from C. Trautwein and M. Manns) was utilized to assay the transcriptional activation by the C/EBP family of proteins. These experiments were performed with cell lines stably transfected with the CRP-CAT reporter construct. Since proper adipocyte differentiation requires that the cells be confluent and since confluence greatly decreases transfection efficiency, reproducible results could be obtained using only stable transfections. Three cell lines were created. One cell line contained CRP-CAT and an empty vector. One contained CRP-CAT and the FBR v-Fos expression vector, and the last contained CRP-CAT and the G2A v-Fos expression vector. In agreement with Fig. 2, the CRP-CAT cells showed normal differentiation as judged by Oil Red O staining (data not shown). Neither the FBR v-Fos + CRP-CAT cells nor the G2A v-Fos + CRP-CAT cells differentiated as shown by Oil Red O staining (data not shown). In addition, upon differentiation, the CRP-CAT cells showed 50-fold higher G3PDH activity when compared with the FBR v-Fos + CRP-CAT cells or the G2A v-Fos + CRP-CAT cells (data not shown). These cells were indistinguishable in their differentiation characteristics from those described in Fig. 2.

To determine whether the induction of C/EBP\( ^b \) created functional protein, we differentiated the CRP-CAT, FBR v-Fos + CRP-CAT and G2A v-Fos cells for 1 and 2 days. Each cell line produced equivalent amounts of C/EBP\( ^b \) protein as shown by Western blotting (data not shown). Since each of these cell lines contained the C/EBP-responsive reporter construct, CRP-CAT, CAT activity reflects C/EBP\( ^b \) activity. CRP-CAT cells showed a 3-fold induction of activity upon expression of C/EBP\( ^b \) at both day 1 of differentiation and day 2 of differentiation (Fig. 5A). Expression of G2A v-Fos had no effect upon C/EBP\( ^b \) activity (3-fold induction at day 1 of differentiation and a 4-fold induction at day 2 of differentiation) (Fig. 5A), while FBR v-Fos actually stimulated C/EBP\( ^b \) activity (5-fold induction at day 1 of differentiation and an 8-fold induction at day 2 of differentiation) (Fig. 5A). Not only is C/EBP\( ^b \) expressed at normal levels in FBR v-Fos and G2A v-Fos cells, but G2A v-Fos cells show normal C/EBP\( ^b \) activity, and FBR v-Fos cells showed increased C/EBP\( ^b \) activity.

The above experiment is complicated by the fact that the parental cell line, NIH-3T3 L1, expresses other members of the C/EBP family, including C/EBP\( ^\delta \). To further study the effect of
FBR v-Fos and G2A v-Fos on C/EBP\(\beta\), we utilized a cell line in which we could more tightly control the expression levels of FBR v-Fos, G2A v-Fos, and C/EBP\(\beta\). Since C/EBP\(\beta\) expression is tissue-specific (50, 51), we utilized a HeLa cell line which does not express any of the C/EBP family members (33). HeLa cells were transfected with the CRP-CAT reporter construct, C/EBP\(\beta\) and varying amounts of FBR v-Fos or G2A v-Fos. CAT assays were performed. Fig. 5B shows that expression of C/EBP\(\beta\) causes an approximately 18–20-fold induction of CRP-CAT. At low doses of FBR v-fos and G2A v-fos (1 \(\mu\)g), these oncproteins stimulate C/EBP\(\beta\) activity slightly (Fig. 5B). At higher doses (5 \(\mu\)g and 10 \(\mu\)g), however, neither expression of FBR v-Fos nor G2A v-Fos at varying doses significantly affects activation of the CRP-CAT reporter construct in the presence of C/EBP\(\beta\) (Fig. 5B).

To further show that FBR v-Fos does not affect C/EBP\(\beta\) activity, C/EBP\(\beta\)’s ability to bind its consensus DNA binding site in the presence and absence of FBR v-Fos was assayed by EMSA. RSV-neo cells and FBR v-Fos cells were subjected to the differentiation protocol for 2 days. Electrophoretic mobility shift assays were performed using equal quantities of nuclear extracts. Both cell lines show compatible binding of C/EBP\(\beta\) to its consensus binding sequence (Fig. 5C, lanes 2 and 5). This binding activity can be supershifted using a C/EBP\(\beta\) antibody, showing that the complex contains C/EBP\(\beta\) (Fig. 5C, lanes 3 and 6). As a control, a C/EBP\(\beta\) antibody had no effect on DNA binding (Fig. 5C, lanes 4 and 7). This series of experiments shows that FBR v-Fos does not affect the expression, the DNA binding ability, or transactivation capability of C/EBP\(\beta\).

We have shown in Fig. 4 that C/EBP\(\alpha\) expression is significantly impaired in these cell lines. To show that this impaired expression has functional significance, the cell lines which contain the C/EBP-responsive reporter, CRP-CAT, were utilized. CRP-CAT cells, FBR v-Fos + CRP-CAT cells, and G2A v-Fos + CRP-CAT cells were differentiated for 5 and 8 days, and CAT assays were performed. Activity of the CRP-CAT construct was significantly higher (Fig. 6A; 5–7 times higher at 5 days of differentiation and 10 times higher at 8 days of differentiation) in the CRP-CAT cells when compared with those cells which expressed either FBR v-Fos or G2A v-Fos. Therefore, the lack of C/EBP\(\alpha\) expression in cells which express FBR v-Fos or G2A v-Fos has functional significance as there is little C/EBP\(\alpha\) activity in these cells.

Since C/EBP\(\alpha\) can up-regulate its own expression (44, 49), if FBR v-Fos and G2A v-Fos inhibited C/EBP\(\alpha\) activity, C/EBP\(\alpha\) could not feed back upon its own promoter to up-regulate its expression. To determine if FBR v-Fos or G2A v-Fos could directly inhibit C/EBP\(\alpha\) activity, we again utilized HeLa cells. HeLa cells were transiently transfected with the CRP-CAT reporter construct, C/EBP\(\alpha\), and varying amounts of FBR v-Fos or G2A v-Fos. In previous work, we have shown that expression of FBR v-Fos and G2A v-Fos does not affect expression from the MSV-driven C/EBP\(\alpha\) construct used for this experiment (33). 48 h after transfection, transfection efficiency was standardized, and CAT assays were performed. Fig. 6B shows that CRP-CAT is not affected by expression of FBR v-Fos or G2A v-Fos in the absence of C/EBP\(\alpha\). Expression of C/EBP\(\alpha\) causes a 50-fold induction of CAT from the CRP-CAT construct. When FBR v-Fos is co-expressed with C/EBP\(\alpha\) at varying doses, C/EBP\(\alpha\)’s activation of CRP-CAT decreases. At the highest amount of FBR v-Fos, CRP-CAT activation by C/EBP\(\alpha\) is approximately 20% of that without FBR v-Fos. G2A v-Fos can also inhibit C/EBP\(\alpha\)'s activation of CRP-CAT (Fig. 6B), so while FBR v-Fos and G2A v-Fos have no effect on either activity or expression C/EBP\(\beta\), both of these oncproteins can inhibit activity and expression of C/EBP\(\alpha\).

The above experiment suggests that expression of these oncproteins decreases C/EBP\(\alpha\) activity, thereby decreasing transcriptional autoregulation of the C/EBP\(\alpha\) gene. To support this hypothesis, Northern analysis on RSV-neo cells, FBR v-Fos cells, and G2A v-Fos cells was performed. The three cell lines were subjected to the differentiation protocol for 8 days, and total RNA was obtained. Northern blotting was performed using a full-length C/EBP\(\alpha\) probe. The relative amounts of RNA are shown in Fig. 7 (lower panel). Only the RSV-neo cells contained C/EBP\(\alpha\) mRNA (Fig. 7, arrow). Neither the FBR v-Fos nor the G2A v-Fos contained C/EBP\(\alpha\) RNA, indicating that FBR v-Fos and G2A v-Fos inhibit C/EBP\(\alpha\) in such a way as to inhibit mRNA production.

C/EBP\(\beta\) Is Expressed in Tumors from FBR v-Fos Mice, but C/EBP\(\alpha\) Is Not Expressed—To obtain more physiological results, we studied expression of C/EBP\(\alpha\) and C/EBP\(\beta\) in mice...
FIG. 5. FBR v-Fos and G2A v-Fos do not decrease activity of C/EBPβ. A, the cells expressing CRP-CAT, FBR v-Fos + CRP-CAT, or G2A v-Fos + CRP-CAT were subjected to the differentiation protocol for either 1 or 2 days. CAT assays were performed on equal amounts of cell lysate. The CRP-CAT cells showed an activation of 2.83 ± 0.08 and 2.49 ± 0.20 at 1 and 2 days of differentiation, respectively. The FBR v-Fos + CRP-CAT cells showed an activation of 5.21 ± 1.17 and 8.63 ± 1.41 at 1 and 2 days of differentiation, and the G2A v-Fos + CRP-CAT cells showed an activation of 2.89 ± 0.72 and 4.32 ± 1.19 at 1 and 2 days of differentiation. B, HeLa cells were transfected with the indicated constructs. β-Galactosidase was used to standardize transfection efficiency, and CAT assays were performed. A representative CAT assay is shown with fold activation and S.E. values calculated from five independent experiments. C, RSV-neo cells and FBR v-Fos cells were subjected to the differentiation protocol for 2 days. EMSA was performed using equivalent amounts of nuclear extract. Lane 1, probe only; lane 2, RSV-neo cell extract; lane 3, RSV-neo cell extract + C/EBPβ antibody; lane 4, RSV-neo cell extract + C/EBPα antibody; lane 5, FBR v-Fos cell extract; lane 6, FBR v-Fos cell extract + C/EBPβ antibody; lane 7, FBR v-Fos cell extract + C/EBPα antibody.
transgenically expressing FBR v-Fos (5). Immunohistochemical staining for C/EBPα and C/EBPβ was performed on liver sections and tumor sections from mice transgenically expressing FBR v-Fos. A negative control of staining with serum shows limited background in a tumor section (Fig. 8A). The liver normally contains high amounts of both C/EBPα and C/EBPβ (50, 51), so liver sections were used as a positive control to show staining of C/EBPα and C/EBPβ. Liver sections from these mice show nuclear staining of both C/EBPα and C/EBPβ, indicating that FBR v-Fos does not affect expression of these proteins in tissues other than adipocytes (Fig. 8, B and C). Tumor sections from these mice show the same expression pattern of C/EBPα and C/EBPβ as is seen in culture. C/EBPβ is expressed (Fig. 8D), but C/EBPα is not expressed (Fig. 8E). The fact that tumors caused by FBR v-Fos show similar C/EBPα and C/EBPβ expression patterns to that seen in culture implies that FBR v-Fos is also inhibiting adipogenesis in vivo by inhibiting expression and activity of C/EBPα.

DISCUSSION

In this work, we have shown that FBR v-Fos inhibits the morphological (Fig. 2), biochemical (Table I), and growth (Fig. 3) characteristics of terminally differentiated adipocytes. None of this inhibition is due to the myristylation state of FBR v-Fos as the nonmyristylated FBR v-Fos mutant, G2A v-Fos, also caused inhibition of adipocyte differentiation (Figs. 2 and 3; Table I). Both FBR v-Fos and G2A v-Fos allow the initiation of adipogenesis to occur as C/EBPβ is induced at normal levels in these cells. However, the later stages of adipogenesis are faulty as C/EBPα, a critical regulator of adipogenesis, is expressed (Figs. 4 and 7). In addition, we have shown that both FBR v-Fos and G2A v-Fos inhibit C/EBPα activity without affecting C/EBPβ activity (Figs. 5 and 6). Finally, these results have in vivo significance as liposarcomas from FBR v-Fos transgenic mice show a similar C/EBP expression pattern as that seen in culture (Fig. 8). These results imply that FBR v-Fos causes liposarcomas in mice through its action on the C/EBPα.

FBR v-Fos' induction of carcinogenesis can be divided into two steps. One step is the initiation of carcinogenesis, and the other step is the progression of that carcinogenesis. Only the second step, that of progression, is myristylation-dependent. Our laboratory has previously shown that FBR v-Fos causes cells to lose the capacity to repair damage caused by ionizing radiation (6). For this reason, these cells are more likely to suffer DNA damage, leading to an accumulation of cancer-promoting mutations. This sensitivity to radiation is due to the myristylation of FBR v-Fos as cells which express G2A v-Fos show none of FBR v-Fos' radiation effects (6). Since mice which express FBR v-Fos develop malignant liposarcomas that kill the animal and mice which express G2A v-Fos develop benign lipomas, mice which express FBR v-Fos are more likely to undergo the progression from a benign tumor to a malignant tumor, and this progression is most likely due to the genomic
and G2A v-Fos initiation of carcinogenesis. Tumors arising from the FBR v-Fos mice, both FBR v-Fos and G2A v-Fos have similar effects on the comas. This is an attractive hypothesis because it has been inhibitor of C/EBP-mediated transactivation can cause liposarcomas (28). The implication is that deregulation of an (25, 26). This CHOP-Ews fusion protein is sufficient to trans-

tion is an important prognostic indicator (1), the study of the effects of FBR v-Fos and G2A v-Fos on adipogenesis is an important model system for the study of both the initiation of tumors and the differentiation state of tumors. The results of this work have implicated the C/EBP family of proteins in the formation of liposarcomas. That the C/EBP family of proteins is targeted for liposarcomas formation by FBR v-Fos and G2A v-Fos is not unexpected. Work in other laboratories has previously implicated this family of proteins in the formation of liposarcomas (25–28). CHOP, a member of the C/EBP family, can bind as a heterodimer to various C/EBP proteins, but this heterodimer cannot transactivate at C/EBP-responsive sites (24). In a myxoid liposarcoma, CHOP was found in fusion via a chromosomal translocation to the Ews RNA-binding protein (25, 26). This CHOP-Ews fusion protein is sufficient to transform fibroblasts (28). The implication is that deregulation of an inhibitor of C/EBP-mediated transactivation can cause liposarcomas. This is an attractive hypothesis because it has been shown that C/EBPα is a potent suppresser of cell division (19, 20, 22, 46). As such a potent antimitotic agent, inhibition of either expression or activity of C/EBPα would be necessary to allow a cell to continue in the growth pool and thereby increase the likelihood of carcinogenesis occurring.

Exactly how FBR v-Fos inhibits C/EBPα expression and activity is unknown. Results from other laboratories have also shown an antagonistic relationship between the Fos proteins and the C/EBP family of proteins. For instance, c-Fos has been shown to antagonize C/EBPβ by directly binding it (52). This mechanism of repression cannot be the case in our system because FBR v-Fos has no effect on C/EBPβ's DNA binding or transactivating capability. In addition, we have been unable to detect FBR v-Fos binding to C/EBPβ either in vitro or in vivo, and previous results in our laboratory have shown that FBR v-Fos does not bind C/EBPα (33). While c-Fos may bind to the C/EBP family of proteins to affect C/EBP signaling directly (52), FBR v-Fos does not share this ability, and the repression of C/EBPα by FBR v-Fos must be occurring in another manner.

The antagonism of C/EBP family of proteins and c-Fos has also been shown by another laboratory in a cell signaling context. c-Fos has been shown to repress phosphoenolpyruvate carboxykinase (PEPCK) gene expression, a process driven in the liver by the C/EBP family of proteins (52). It was hypothesized in this study that the repression by c-Fos could represent the result of multiple signaling pathways converging on the PEPCK gene (53). c-Fos could be part of a signaling pathway whose function is to antagonize the C/EBP family of proteins and ensure proper gene expression for the environment to which the cell is exposed. In this manner, expression of FBR v-Fos in the pre-adipocyte may activate alternative signaling pathways which are inconsistent with adipocyte differentiation and C/EBPα activity. This hypothesis is consistent with the fact that cells which express FBR v-Fos or G2A v-Fos do not undergo the growth arrest characteristic of terminally differentiated cells.

The characteristics that FBR v-Fos and G2A v-Fos impart on the preadipocyte are consistent with the deregulation of the C/EBP family of proteins which must occur to form a liposarcoma. These v-Fos oncproteins cause an inhibition of the expression of C/EBPα. They can inhibit the activity of C/EBPα, and they subvert the C/EBPα-mediated growth arrest. In addition, the liposarcomas from FBR v-Fos-expressing mice show no C/EBPα expression. For these reasons, this work strongly implies that these v-Fos oncproteins induce lipomas and liposarcomas through C/EBP inhibition, and this work further establishes the C/EBP family's role in the formation of liposarcomas. By using a model system in which a retroviral oncogene inhibits fat cell differentiation, this work helps to begin to define the molecular mechanism by which differentiation and cancer are linked.

Acknowledgments—We thank Drs. Christian Trautwein and Michael Manns (Medizinische Hochschule Hannover) for the CRP-CAT plasmid. We also thank Marilyn E. Thompson for critical comments on this manuscript and Annette Hall for expert technical assistance.

REFERENCES

1. Cotran, R. S., Kumar, V., and Robbins, S. L. (1994) Pathological Basis of Disease, pp. 153–200, W. B. Saunders, Philadelphia
2. Finkel, M. P., Reilly, C. A., Jr., and Biskis, B. O. (1975) Front. Radiat. Ther. Oncol. 10, 28–39
3. Lee, C. K., Chan, E. W., Reilly, C. A., Pahnke, V. A., Rockus, G., and Finkel,

2 D. W. Abbott and J. T. Holt, unpublished observations.
| Reference                                                                 | Page Numbers     |
|--------------------------------------------------------------------------|------------------|
| Green, H., and Kehinde, O. (1976) Cell  Proc. Natl. Acad. Sci. U. S. A. 11, 193–202 |
| Russell, T. R., and Ho, R.-J. (1976) Annu. Rev. Biochem.                 | 626–636          |
| Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994) Genes Dev. 18, 296–309 |
| Yeh, W.-C., and McKnight, S. L. (1995) Curr. Opin. Cell Biol. 7, 135–146 |
| Samuelsson, L., Stromberg, K., Vikman, D, Bjursell, G., and Enerback, S. (1991) EMBO J. 10, 3787–3793 |
| Christy, R. J., Yang, V., Ntambi, J., Geiman, D., Landschulz, W. H., Friedman, A., Nakabeppe, Y., Kelly, T., and Lane, M. D. (1989) Genes Dev. 3, 1323–1335 |
| Park, E. A., Roessler, W. J., Liu, J., Klemm, D. J., Gurney, A. L., Thatcher, J. D., Shuman, J. D., Friedman, A., and Hanson, R. W. (1990) Mol. Cell. Biol. 10, 6264–6272 |
| Cheneval, D., Christy, R. J., Geiman, D., and Lane, M. D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8465–8469 |
| Freytag, S., Paelli, D. L., and Gilbert, J. D. (1994) Genes Dev. 8, 1654–1663 |
| Lin, F.-T., and Lane, M. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8757–8761 |
| Hendricks-Taylor, L. R., and Darlington, G. J. (1995) Nucleic Acids Res. 23, 4726–4733 |
| Timchenko, N., Wilde, M., Nakamichi, M., Smith, J. R., and Darlington, G. J. (1996) Genes Dev. 10, 804–815 |
| Ron, D., and Habener, J. (1992) Genes Dev. 6, 439–453 |
| Czecat, A., Aman, P., Mandahl, N., and Ron, D. (1993) Nature 363, 640–644 |
| Rabbits, T. H., Forster, A., Larson, R., and Nathan, P. (1996) Nat. Genet. 4, 175–180 |
| Batchvarova, N., Wang, X. Z., and Ron, D. (1995) EMBO J. 14, 4654–4661 |
| Zinzsner, H., Albatal, R., and Ron, D. (1994) Genes Dev. 8, 2517–2526 |

**FBR v-Fos Inhibits Adipocyte Development**

Hunter, T. (1997) Cell 8, 333–346

Graham, P., and van der Eb, A. (1973) Virology 52, 456–467

Kamata, N., Jotte, R. M., and Holt, J. T. (1991) Mol. Cell. Biol. 11, 765–772

Kamata, N., and Holt, J. T. (1992) Mol. Cell. Biol. 12, 876–882

Jotte, R. M., Kamata, N., and Holt, J. T. (1994) J. Biol. Chem. 269, 16383–16396

Treutlein, C., Walker, D. L., Plumpe, J., and Manns, M. P. (1995) J. Biol. Chem. 270, 15130–15136

Yeh, W.-C., Cao, Z., Classon, M., and McKnight, S. L. (1995) Genes Dev. 9, 168–181

Kuzak, L. P., and Jensen, J. T. (1974) J. Biol. Chem. 249, 7775–7781

Wise, L. S., and Green, H. (1979) J. Biol. Chem. 254, 273–275

Kerr, L. D., Holt, J. T., and Matrisian, L. M. (1988) Science 242, 1424–1427

Rana, B., Xie, Y., Mischoulon, D., Bucher, N. L., and Farmer, S. R. (1995) J. Biol. Chem. 270, 18123–18132

Serrero, G., and Mills, D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3912–3916

Serrero, G., and Legak, N. (1996) Int. J. Obes. 3, 58–64

Umek, R., Friedman, A., and McKnight, S. L. (1991) Science 251, 288–292

Descombes, P., and Schilcher, U. (1991) Cell 67, 569–579

Chrisry, R. J., Kaestner, K. H., Geiman, D. E., and Lane, M. D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2593–2597

Chawla, A., and Lazar, M. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1786–1790

Wu, Z., Xie, Y., Bucher, N. L., and Farmer, S. R. (1995) Genes Dev. 9, 2350–63

Timchenko, N., Wilson, D. R., Taylor, L. R., Wilde, A. M., Abdelsayed, S., Sawadogo, M., and Darlington, G. J. (1995) Mol. Cell. Biol. 15, 1192–1202

Wu, Z., Bucher, N. L., and Farmer, S. R. (1996) Mol. Cell. Biol. 16, 4128–4136

Legraverend, K., Antenson, P., Fobloty, P., and Zantopoulos, K. G. (1993) Nucleic Acids Res. 21, 1735–1742

Johnson, P. F, Landschulz, W. H., Friedman, A., and McKnight, S. L. (1989) Genes Dev. 2, 1146–1156

Birkenmeier, E., Gwynn, B., Howard, S., Jerry, J., Gordon, J., Landschulz, W. H., and McKnight, S. L. (1995) Genes Dev. 1, 133–146

Hsu, W., Kerppola, T. K., Chen, P.-L., Curran, T., and Chen-Kiang, S. (1994) Mol. Cell. Biol. 14, 268–276

Gurney, A. L., Park, E. A., Giralt, M., Liu, J., and Hanson, R. W. (1992) J. Biol. Chem. 267, 18133–18139