

Loss of tumorigenicity of rat glioblastoma directed by episome-based antisense cDNA transcription of insulin-like growth factor I

(tumor/extrachromosomal replication/immunoregulation)

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Communicated by Emanuel Margoliash, February 26, 1992 (received for review July 18, 1991)

ABSTRACT Malignant glioma is the most common brain tumor. The molecular basis of glioma tumorigenicity has not been defined. Cultured glioma cells accumulate high levels of insulin-like growth factor I (IGF-I) transcripts. We asked whether IGF-I expression is coupled to tumorigenicity, using a combined *in vivo/in vitro* system employing antisense RNA for IGF-I. An antisense IGF-I expression construct in an expression vector that incorporates Epstein–Barr virus replicative signals and the ZnSO₄-inducible metallothionein I transcriptional promoter was assembled. Stable glioma transfectants were derived from C6 glioma cells, which constitutively express IGF-I. B-104 neuroblastoma cells, derived originally from the same tumor but not expressing IGF-I, were also transfected as controls. In the absence of ZnSO₄, the C6 transfectants expressed high levels of IGF-I mRNA and protein as detected by *in situ* hybridization and immunocytochemistry, respectively. Addition of ZnSO₄ in the culture medium resulted in high levels of antisense transcript accumulation and dramatically decreased levels of endogenous IGF-I mRNA and IGF-I protein. Subcutaneous injection of either nontransfected C6 parental cells or C6 cells transfected with vector without IGF-I sequences into rats resulted in large tumors after 2 weeks, as did transfected and nontransfected B-104 cells. However, the rats injected with transfected C6 cells yielded no tumors after 40 weeks of observation. Two weeks after injection of the transfected C6 cells a small cyst was apparent in six rats. Histologic sections revealed a few glioma cells infiltrated by a large number of mononuclear cells. No infiltration of mononuclear cells was apparent in the glioma tumors resulting from injection of parental (nontransfected) cells, suggesting that the parental cells, but not the antisense IGF-I transfectants, escape the host immune response.

Malignant gliomas are the most common primary brain tumor, accounting for 29% of all primary brain tumors in humans (1). The median survival rate is <1 year and the 5-year survival rate is <5.5% (2–4). The molecular basis of the tumorigenicity of glioma cells has not been defined.

We (5) and others (6) have reported that the rat glioma cell line C6 expresses high levels of insulin-like growth factor I (IGF-I), which is enhanced when grown in serum-free medium. Therefore, IGF-I expression may be coupled to cellular proliferation and tumorigenicity. Since C6 cells express IGF-I and IGF-II receptors (6), an autocrine loop involving IGF-I may exist and drive the tumorigenic phenotype. Until now, the autocrine hypothesis of malignant transformation has derived experimental support primarily from antibody blocking (7) and sense gene transfer studies. However,

antibody-blocking is ineffective for the analysis of intracellular autocrine loops (7) and antibodies vary considerably in their blocking efficacy. In principle, mutagenesis strategies offer more definitive and unambiguous approaches for analyzing autocrine mechanisms in tumor cells.

Classically, in order to assign a functional role of a particular gene product, one relies on naturally occurring mutants. The *in vitro* generation of cellular mutants has limited applicability due to the diploid nature of most genes and to the lack of adequate mutant selection. The use of antisense RNA expression generates phenocopies of a null mutation for IGF-I. Inhibition of IGF-I by antisense provides a direct approach for assessing its autocrine contribution to the tumorigenic phenotype of C6 cells *in vitro* and *in vivo*. According to the antisense concept, an antisense transcriptional cassette is used to generate a sufficiently high concentration of complementary RNA to a given endogenous mRNA species; this antisense RNA will either hybridize to the endogenous mRNA or disrupt its transcription or processing, thereby preventing the synthesis of protein product (8). Here we report the application of the antisense approach, in a way that allows for high levels of antisense RNA transcription. Our vector includes the Epstein–Barr virus (EBV) replicative signals to permit self-replication and to maximize antisense transcript levels. Moreover, this report demonstrates a successful use of cells engineered to express antisense for subsequent functional analyses *in vitro* and *in vivo*. This combined *in vitro/in vivo* system can be used in a more general way to screen the transformation potential of selected genes.

In this study, we provide a demonstration of a transformation role for IGF-I, highlighting the fact that transformation phenotypes may arise through unexpected molecular mechanisms. This finding has potential therapeutic implications, raising the possibility of anti-IGF-I therapy for the treatment of gliomas.

MATERIALS AND METHODS

Cell Culture. Rat C6 glioma cells were obtained from the American Type Culture Collection and cultured essentially as described by Kiess *et al.* (6). B-104 neuroblastoma cells, originating from the same tumor as the C6 glioma line and serving as a control, were a gift from Lloyd Culp, in our school. Culture medium for transfected cells was supplemented with 0.2 mg of hygromycin per ml (Calbiochem) to maintain selection pressure up until 10 days before subcutaneous injection into rats (male DB-X; age, 3 months; 10⁷ cells

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Abbreviations: IGF-I, insulin-like growth factor I; EBV, Epstein–Barr virus; GFA, glial fibrillary acidic protein.

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per injection). Twenty-four hours before injection cells were washed and maintained in serum-free medium.

Antibodies. Polyclonal antibodies against human IGF-I were provided by the National Hormone and Pituitary Program or obtained commercially from KabiGen (Stockholm). Immunocytochemical localization of IGF-I by peroxidase was carried out as described earlier (9). As a positive control, sectioned adult rat liver was employed; as a negative control, human and mouse fibroblast cell lines (HT-1080 and LTK, respectively) were employed. The IGF-I localized immunocytochemically was evaluated semiquantitatively by plotting the "end points" of staining; these end points were defined as maximal dilutions of antibodies still giving a positive reaction in the cells. The end point of staining was obtained at a dilution of 1:2000 to 1:3000; the limit of sensitivity occurred at a dilution of 1:100–1:200 (positive nonspecific reaction). The fluorescein isothiocyanate method was carried out by standard procedures using commercially available second antibodies (goat antirabbit) conjugated to fluorescein isothiocyanate. The limit of sensitivity was 1:20–1:30 dilution. For all photographs, the antibody dilution used was 1:400–1:500.

Hybridization. *In situ* hybridization was carried out as we recently described in detail (10, 11) except that ethanol was omitted in the prehybridization as well as in the final wash steps. Details are given in the legends to the figures. Transfer blot hybridization was carried out as described previously (5, 12, 13).

Transfection. Transfection of C6 glioma and B-104 neuroblastoma cells was accomplished using Lipofectin reagent (GIBCO/BRL) according to the supplier's instructions.

Construction of the Episome-Based Plasmid pAntiIGF-I. A diagrammatic representation of the steps employed to assemble the vector is shown in Fig. 1. The pMK' plasmid, containing the mouse metallothionein I promoter fused to herpes simplex thymidine kinase was kindly provided by Richard Palminter (14). pIGF-I harboring a human hepatic cDNA for IGF-I was kindly provided by Martin Jansen (15). pUC18 (16) was obtained from Peter Harte, in our school. pBluescript KS⁺ (Stratagene) was obtained commercially. We described the construction of REP-4 elsewhere (17). REP-4 contributes two genes from the EBV to pAntiIGF-I, the origin of replication (EBV ori-P) and the EBV-encoded nuclear antigen I, which drives the EBV ori-P's replicative function. These two genes allow pAntiIGF-I to replicate as an episome. The simian virus 40 poly(A) signal is present as a termination signal for transcription.

RESULTS

Stable transfectants of C6 glioma cells were derived and were transferred to serum-free medium for 24 hr. We have shown that in serum-free medium, expression of IGF-I transcripts is greatly enhanced (5). Fig. 2 depicts an autoradiogram from transfer blots of 10 μg of total RNA of transfected and untransfected C6 and B-104 cells exposed to serum-free medium. The pattern of IGF-I transcripts in parental and transfected cells matches that published by us and others (6) showing prominent bands at 7.5, 1.8, and 1 kilobase (kb). Treatment with 50 μM ZnSO₄ (Fig. 2a, lane 3) clearly induces strong expression of the 1-kb antisense transcript as well as results in almost complete disappearance of the endogenous IGF-I transcripts at 7.5 and 1.8 kb. Rehybridization of the same blot with a single-stranded complementary RNA indicated that the endogenous 1-kb transcript also disappeared. When the same blot was probed with human nick-translated, double-stranded IGF-I cDNA, the endogenous rat IGF-I transcript was not apparent (Fig. 2a, lanes 4 and 5). Rehybridization of the blot with a single-stranded complementary RNA probe indicated that the 1-kb transcript in lane 4 was

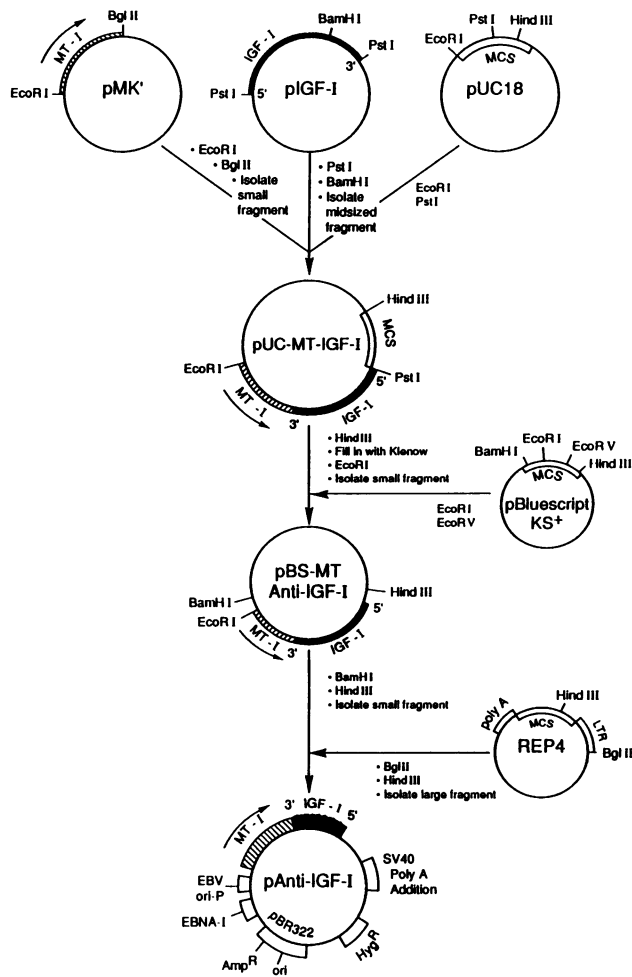


FIG. 1. Diagrammatic representation of the steps employed to construct the episomal vector pAntiIGF-I. MT-I, metallothionein I; MCS, multiple cloning site; LTR, long terminal repeat; EBNA-I, EBV-encoded nuclear antigen I; SV40, simian virus 40; Amp^R, ampicillin resistance gene; Hyg^R, hygromycin resistance gene.

background antisense expression. Untransfected B-104 cells show no detectable IGF-I transcripts (Fig. 2b, lane 1),

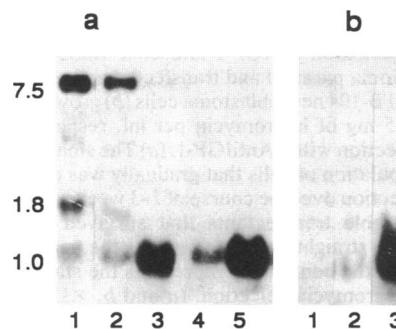


FIG. 2. Induction of antisense transcripts by ZnSO₄ in cultured C6 glioma and B-104 neuroblastoma cells. The figure is a composite of several RNA transfer blots. Ten micrograms of total RNA was used per lane. Molecular sizes (in kilobases) of the major IGF-I transcripts are shown on the left. (a) Lane 1, parental nontransfected C6 glial cells exposed to serum-free medium. Lanes 2 and 3, transfected C6 glioma cells incubated in serum-free medium in the absence (lane 2) or presence (lane 3) of ZnSO₄. For lanes 2 and 3, nick-translated rat IGF-I cDNA was used. Lanes 4 and 5, the blot shown in lanes 2 and 3 was rehybridized to nick-translated, human IGF-I cDNA. (b) Lane 1, untransfected B-104 neuroblastoma cells. Lanes 2 and 3, transfected B-104 neuroblastoma cells incubated in the absence (lane 2) or presence (lane 3) of ZnSO₄.

whereas transfected B-104 cells show a strong induction of antisense transcripts in cells treated with ZnSO₄ (compare lane 3 with lane 2).

Stable transfection resulted in change in morphology of C6 glioma cells (Fig. 3*a*) and B-104 neuroblastoma cells (Fig. 3*b*). Parental C6 cells react positively with antibody to IGF-I by the immunoperoxidase procedure in the presence (Fig. 3*d*) or absence (Fig. 3*c*) of ZnSO₄. In contrast, B-104 neuroblastoma cells show no staining reaction with the above procedure (Fig. 3*e* and *f*). However, in the presence of ZnSO₄ the immunostaining disappeared almost completely (Fig. 3*h*) from the transfected glioma cells. *In situ* hybridization with single-stranded ³⁵S-labeled oligonucleotide showed that transfected glioma cells treated with ZnSO₄ express antisense transcripts (Fig. 3*i* and *j*). However, without ZnSO₄ no hybridization signal is apparent, using the same cells and probe at the same specific activity (Fig. 3*k* and *l*). As a control, transfected B-104 neuroblastoma cells gave similar results (Fig. 3*m-p*).

Fig. 4 further demonstrates that induction of antisense IGF-I transcripts results in disappearance of IGF-I protein. The green immunofluorescence indicating the presence of IGF-I in transfected cells (Fig. 4*a*) disappears when cells are treated with ZnSO₄ (Fig. 4*b*). Fig. 4*c-e* shows the same microscopic field in different optical conditions and depicts ZnSO₄-treated cells with no immunofluorescent staining (Fig.

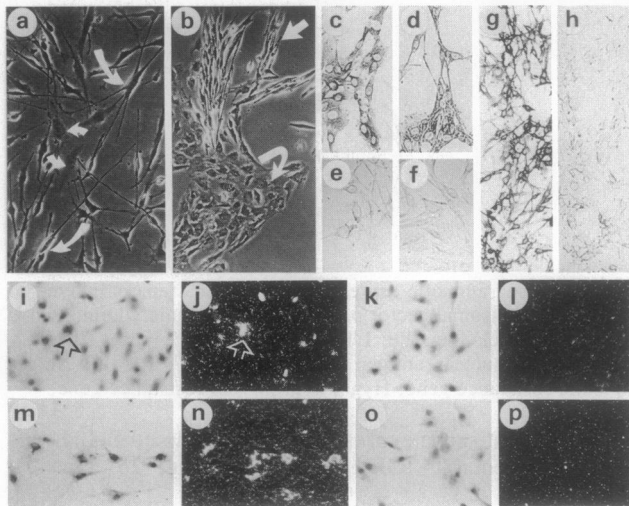


FIG. 3. Localization of IGF-I antisense transcripts and IGF-I protein in C6 glioma parental and transfected cells. (*a* and *b*) Rat C6 glioma cells (*a*) and B-104 neuroblastoma cells (*b*) grown in the presence of 0.2 mg or 0.5 mg of hygromycin per ml, respectively, 2 weeks following transfection with pAntiIGF-I. (*a*) The small arrows indicate the parental population of cells that gradually was eliminated during hygromycin selection over the course of 2–3 weeks. The large arrows represent the stable transfectants that survived the hygromycin selection. (*b*) The straight arrow represents the parental population of cells as above; the bent arrow represents the stable transfectants surviving the hygromycin selection. (*a* and *b*, $\times 120$.) (*c-h*) Immunocytochemical labeling of cultured cells with antibodies to human IGF-I using a quantitative immunoperoxidase technique (18). (*c* and *d*) Positively stained parental C6 incubated in the absence (*c*) or presence (*d*) of 50 μ M ZnSO₄. (*e* and *f*) Negative reaction obtained with B-104 neuroblastoma cells incubated in the absence (*e*) or presence (*f*) of ZnSO₄. (*g* and *h*) C6 glioma transfected cells incubated in the absence (*g*) or presence (*h*) of ZnSO₄. (*c-f*, $\times 120$; *g* and *h*, $\times 70$.) (*i-p*) *In situ* hybridization of cultured cells with ³⁵S-labeled human sense oligonucleotides to detect antisense transcripts. (*i-l*) C6 transfected cells maintained in the presence (*i* and *j*) or absence (*k* and *l*) of ZnSO₄. The arrow points out an example of a cell demonstrating a positive signal. In *k* and *l*, only background hybridization is apparent. (*m-p*) B-104 transfected cells maintained in the presence (*m* and *n*) or absence (*o* and *p*) of ZnSO₄. (*i, k, m*, and *o*, bright field; *j, l, n*, and *p*, dark field. *i-p*, $\times 120$.)

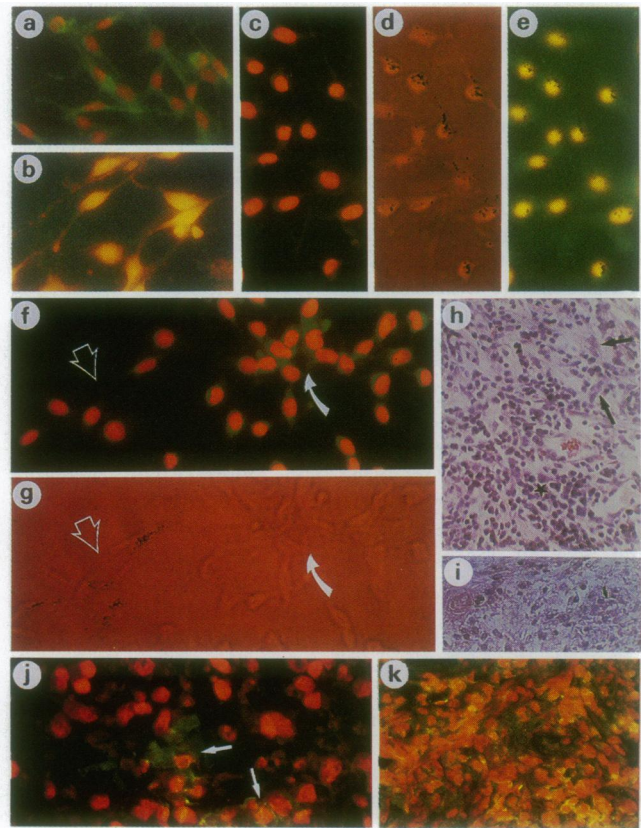


FIG. 4. Simultaneous localization of IGF-I antisense transcripts and staining of IGF-I protein in glioma cells *in vitro* and immunocytochemical identification of glioma cells *in vivo*. (*a* and *b*) Immunocytochemical labeling of C6 transfected cells with anti-IGF-I antibodies using an indirect immunofluorescence technique. Note the presence of IGF-I (green fluorescein isothiocyanate fluorescence) in *a* and the absence of fluorescence in cells treated with ZnSO₄ in *b*. The nuclei are counterstained with *p*-phenylenediamine (19). (*a* and *b*, $\times 120$.) (*c-e*) C6 transfected cells maintained in the presence of ZnSO₄ for 8–10 hr. *c-e* represent the same microscopic field. Antisense transcripts were localized by *in situ* hybridization, as in Fig. 3, and were then subjected to immunofluorescent labeling of IGF-I protein. The absence of protein (*c*) is accompanied by a striking *in situ* hybridization signal localized primarily in the nuclei (*d* and *e*). (*d*, bright field; *e*, bright field combined with UV lamp.) (*f* and *g*) Mixed culture of C6 glioma parental and transfected cells maintained for 8–10 hr in the presence of ZnSO₄. The same microscopic field is visualized in *f* and *g*. Cells showing positive immunofluorescent labeling for IGF-I (*f*, solid arrow) show no detectable signal for IGF-I antisense transcripts (*g*, solid arrow), whereas cells showing a strong positive signal for antisense transcripts (*g*, open arrow), presumably the transfected cells, show no detectable fluorescent staining for IGF-I (*f*, open arrow). See also Fig. 3. (*c-g*) Nuclei are counterstained with *p*-phenylenediamine. (*h-k*) Histologic sections of injected glioma cells. (*h*) Section (hematoxylin/eosin) of a lesion observed at the point of injection of C6 glioma transfected cells and removed 3 weeks after cell injection. Mononuclear cells, the majority of them plasmocytes and macrophages (star), as well as lymphocytes are observed in the organizing tissue surrounding a few glial cells (arrows). (*i*) Histological section of a small area of a tumor induced by injection of C6 glioma cells (nontransfected). Note dispersed small cells of astrocytic type, some of them forming a pseudorosette (arrow). (*h* and *i*, $\times 95$.) (*j* and *k*) Immunofluorescence staining of glial fibrillary acidic protein (GFA, used for identification of glial cells), in the lesion produced by injection of C-6 transfected cells (*j*) and in the tumor developed after injection of nontransfected cells (*k*). (*j*) Note GFA-positive cells (green fluorescence, arrows) surrounded by mononuclear cells (visible in *h*). (*k*) An area of glioblastoma showing positive reaction with anti-GFA antibodies. The nuclei in *j* and *k* are counterstained with *p*-phenylenediamine. (*h* and *i*, $\times 95$; *j*, $\times 190$; *k*, $\times 120$.)

4c). The same cells hybridize strongly to ^{35}S -labeled sense oligonucleotides (Fig. 4d), which are localized in cell nuclei (Fig. 4e). Variation of the position of silver grains with respect to the microscope focal plane accounts for the apparent lack of grains over some cells in Fig. 4d and e. Of special note are the results shown in Fig. 4f and g, which show a mixed culture of transfected and parental C6 cells maintained together in the presence of ZnSO_4 , stained for IGF-I protein (Fig. 4f) and also hybridized *in situ* to ^{35}S -labeled sense oligonucleotides (Fig. 4g). Only transfected cells not showing immunofluorescent staining for IGF-I protein manifest RNA antisense hybridization signals in the mixed culture (compare cells near open arrow, which lack the green stain seen in the cell cluster, and isolated cells near closed arrow).

Southern blots of restriction enzyme-digested DNA showed that the patterns of hybridizing fragments in the DNA from transfected cells were similar to those of the purified plasmid used for transfection. The results suggest that pAnti-IGF-I vector DNA was maintained as an episome (data not shown). Such experiments suggested an estimate of 10 episome copies per cell.

The tumorigenicity of C6 glioma cells is evident in the results shown in Fig. 5. Ten rats were injected subcutaneously with 10^7 nontransfected glioma cells above the right hind leg. Each of the 10 rats developed a large tumor. An additional 11 rats were injected subcutaneously with 10^7 transfected glioma cells in the same location. None of the rats injected with transfected cells developed a tumor during the 40 weeks of observation (an additional group of rats has been tumor free for 3 months). The size of the tumor developed 3 weeks after injection of the parental glioma cells is shown in Fig. 5a (arrow), and a representative rat injected with transfected glioma cells is shown on the right. An additional 5 rats were injected with C6 cells that had been transfected with expression vector that did not contain IGF-I sequences. All 5 of these rats developed tumors after 2 weeks.

Fig. 5b depicts a rat injected with 10^7 stably transfected B-104 neuroblastoma cells as a control. In 2 weeks a large tumor was apparent in each of the 10 rats injected (Fig. 5b).

Six of 11 rats injected with the transfected C6 glioma cells developed a small lesion (<1 cm) at the point of injection a few days after injection. Three were removed for further analysis and the others disappeared after 2–3 weeks. A histological section of one of these lesions, stained with hematoxylin/eosin, is shown in Fig. 4h. It shows a few glial cells (positively identified by immunostaining for GFA in Fig. 4j) surrounded by an organizing tissue containing many mononuclear cells, mainly plasmocytes and lymphocyte-like

cells. The other two lesions were histologically identical. This suggests that the host immune system reacted against the transfected glioma cells. By contrast, the tumors resulting from injection of nontransfected glioma cells (which stained positively for GFA; Fig. 4k) did not show any apparent infiltration of mononuclear cells within the tumor (Fig. 4i).

DISCUSSION

We report here a combined *in vitro* and *in vivo* functional analysis of genetically engineered tumor cells corresponding to antisense phenocopies of an IGF-I null mutation. We obtained efficient antisense RNA-mediated inhibition of IGF-I expression in C6 cells using an *in vitro* inducible antisense RNA transcriptional system. An apparent complete shutoff of the protein product was observed following antisense induction *in vitro*. IGF-I inhibition *in vitro* resulted in loss of tumorigenicity *in vivo*. Taken together, the *in vitro* and *in vivo* observations provide clear support of the autocrine hypothesis of tumorigenesis and a demonstration of an unexpected autocrine role for IGF-I in this tumor type.

Antisense strategy has been applied successfully to a growing set of genes in cultured cells and in transgenic animals (20, 21). However, the antisense approach has frequently been complicated by incomplete inhibition of gene expression (21, 22). Graded functional effects are difficult to interpret. Most failures are probably a consequence of insufficient antisense RNA levels and inadequate suppression of the encoded protein product. Integrating vectors, used for most of antisense experiments to date, fail to provide for gene amplification and high-level accumulation of antisense RNA. Moreover, randomly integrating vectors are subject to local regulatory effects exerted by neighboring sequences at sites of integration. Hence, antibiotic-resistant cells may at times express only limited amounts of antisense RNA.

To bypass the above limitations, we employed an episome-based vector that incorporates EBV replicative signals (23). We first showed that such a vector can be used in human T-cell clones for effective (>95%) inhibition of the T-lymphocyte surface molecule CD8 (24). This has been confirmed by others (25, 26). However, we used an inducible promoter for inducible antisense transcription *in vitro*. The low basal activity *in vitro* of the episome-based metallothionein I promoter observed by us contrasts with the higher basal activity previously noted by us and others (27) and is likely to be a consequence of the serum-free environment. The metallothionein I promoter also includes glucocorticoid and cyclic AMP response elements. In contrast to previous studies that were performed in human cell lines, this study involved a rodent cell line. A previous report failed to show EBV episome maintenance in several murine cell lines (23).

The antisense IGF-I transcripts that appeared following ZnSO_4 induction were prominently localized to the nuclei by *in situ* hybridization. This finding supports an intranuclear mechanism for antisense RNA-mediated inhibition, in agreement with observations made by others for several other genes (28–31).

Natural antisense RNA is now known to control the expression of a growing number of eukaryotic genes, including genes related to tumorigenesis (32–41). Hence, our experimental strategy using genetically introduced antisense RNA may mimic natural mechanisms. Recently, antisense RNA complementary to retroviral packaging protein sequences was used to completely suppress Moloney murine leukemia virus-induced leukemia in transgenic mice, pointing to a possible therapeutic use of antisense (40). Our study differs from these previous reports in two important ways. First, we have evaluated tumorigenicity in an immunologically intact animal background. Second, our study focuses on a cytokine (IGF-I) with potential autocrine function.

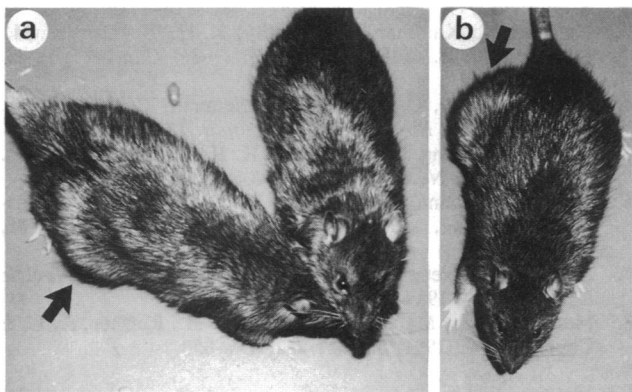


FIG. 5. Tumors derived from C6 glioma nontransfected cells (a, left) and transfected B-104 neuroblastoma cells (b). Arrows indicate the positions of the tumors. The rat on the right in a was injected with C6 glioma transfected cells and did not develop a tumor.

A number of earlier studies support a role for autocrine stimulation in tumor formation, in that anti-cytokine and/or anti-cytokine receptor antibodies can block tumor cellular proliferation *in vitro* (7). However, antibody blocking or oligonucleotide antisense can have limitations in the context of *in vivo* analyses. Expression of DNA constructs resulting in antisense RNA provides a direct and unambiguous experimental approach for studying extracellular and intracellular autocrine loops. Our use of stable antisense transfectants allows analysis of their phenotypic (including tumorigenic) properties *in vitro* and *in vivo* and provides an *in vitro/in vivo* assay system suitable for screening transformation potential of selected genes and particularly useful for dissecting autocrine mechanisms.

Antibody blocking has suggested an extracellular autocrine role for IGF-I in human lung (42) and breast (43) carcinoma cell lines as well as a human osteosarcoma cell line (44).

The mechanism underlying the loss of tumorigenicity for the antisense IGF-I transfectants remains to be determined. Interestingly, we noted a striking infiltration of mononuclear cells expressing IgG and IgM surrounding the injected C6 transfected cells *in vivo*. No such infiltrates were apparent for the parental (IGF-I positive) cells in the tumors formed *in vivo*. Therefore, the loss of tumorigenicity may be due, at least in part, to an enhanced host immune response to the IGF-I-negative cells. These findings offer the intriguing possibility for anti-tumor therapeutic strategies based upon modulation of IGF-I or other cytokine expression.

The presence of IGF-I in placenta and the fetus has argued for a role for this cytokine in fetal development (45, 46). Others have suggested a role for developmental genes in tumorigenesis. The direct linking of IGF-I to the tumorigenic phenotype provides support to the developmental gene:tumorigenic phenotype connection.

Expert assistance in cell culture was provided by K. Lewandowska, C. Williams, and R. Goldschmidt. We thank Dr. U. Roessmann for neurohistopathological evaluation of tumor specimens. This work was supported by National Institutes of Health Grants HD-25004 and CA-43703 (Joseph Ilan), HD-18271 (Judith Ilan), and AR-20678 (to the Northeast Ohio Arthritis Center, B.K.B.) and National Cancer Institute Grant CA-47566 (M.T.).

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