

Tumour suppression associated with expression of human insulin-like growth factor II

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Summary Recent circumstantial evidence has implicated Insulin-like growth factor II in the genesis of several tumour types, notably developmental tumours (Scott *et al.*, 1985; Schofield & Tate, 1987; Wilkins *et al.*, 1989). This type of tumour, thought to originate during the defective differentiation of organ precursors (Miereau *et al.*, 1987), often expresses greatly elevated levels of mRNA for IGF-II, a known mitogen for these cells and abundantly expressed in their presumed normal counterparts (Scott *et al.*, 1985; Schofield & Tate, 1987; Gray *et al.*, 1987). It has been proposed that continued, inappropriate expression of this gene drives tumour growth by an autocrine mechanism. In order to examine the potential role of IGF-II in the growth of tumour cells an IGF-II cDNA was introduced into a retroviral expression vector, and used to infect a cloned fibroblast cell line. Expression of IGF-II conferred a degree of serum independence of growth in cell culture, however, when cells were injected into nude mice as subcutaneous grafts, clones expressing IGF-II from the retrovirus were found to have a greatly increased (five fold) latency of sarcoma formation. After a prolonged lag all cell lines eventually gave rise to tumours in which the introduced IGF-II genes had either been lost or inactivated, suggesting that in this system IGF-II acts as a tumour suppressor gene.

The Insulin-like growth factors I and II are peptides of 70 and 67 amino acids respectively which show a wide variety of biological actions on a large range of target cells. These include effects on proliferation as well as the induction and support of differentiation in many foetal and embryonic cell types. In humans the IGF-II gene is maximally active during the foetal period in specific rapidly proliferating cell types, amongst which are fibroblasts, hepatocytes, metanephric blastema cells and cells of the foetal adrenal cortex (Gray *et al.*, 1987; Brice *et al.*, 1989; Han *et al.*, 1987; 1988). Because many of these cell types are known to respond to the growth factor in primary culture (e.g. Hill *et al.*, 1986; 1987) autocrine and paracrine mechanisms have been suggested as the natural mode of action in these tissues. Transcription of the IGF-II gene in the foetus is driven from a trio of distinct promoters which are used in specific ratios in different tissues (Schofield & Tate, 1987; dePachter-Holthuisen *et al.*, 1987; reviewed, Sussenbach, 1989). The basis of these different ratios is unknown, but all three promoters are profoundly suppressed (50–100 fold) as development and histogenesis proceed.

In many naturally occurring and experimental neoplasms the 'foetal' promoters of IGF-II are 'reactivated'. This observation is particularly striking in the case of a group of developmental neoplasms, hepatoblastoma, embryonal nephroblastoma (Wilms' tumour), adrenocortical carcinoma and rhabdomyosarcoma (Scott *et al.*, 1985), but elevated expression is also seen in hepatocellular carcinoma (Su *et al.*, 1989; Cariani *et al.*, 1988), leiomyoma & leiomyosarcoma (Hopener *et al.*, 1988; Daughaday *et al.*, 1988), colon carcinoma, and liposarcoma (Tricoli *et al.*, 1986) together with fibrosarcomas (Schofield *et al.*, 1989). One common feature of all these tumours is that the presumed foetal counterparts of all these cell types make significant quantities of IGF-II mRNA and protein (Han *et al.*, 1988; Hill, 1990) while their normal adult counterparts do not. *In situ* hybridisation analysis of human foetal kidney formation (Brice *et al.*, 1989) supports the view that the expression of high levels of IGF-II by the blastema component of Wilm's tumour reflects the pattern of

gene expression in *bona fide* blastema during the developmental window during which the tumour is thought to arise. Similar arguments may be made for both hepatoblastoma and adrenal cortical carcinoma. (See Schofield 1991 for review).

Because autocrine growth is thought to operate in the normal foetal counterpart it has been suggested that the reactivated or continued expression of the IGF-II gene in these tumours is responsible for driving cell proliferation in an autocrine fashion. In order to test whether IGF-II might act as an autocrine stimulator of tumour growth, we analysed the effects of IGF-II expression on tumour formation by cells able to respond to IGF-II, but whose endogenous genes are silent.

Materials and methods

Cell culture

Balb/c/3T3 fibroblasts (clone A 31) (Todaro & Green, 1963) were obtained from the Sir William Dunn School of Pathology collection, cultured in a 50/50 mixture of Alpha modified Eagles medium/Ham's F12 (Alpha/Ham) supplemented with 10% heat inactivated foetal calf serum (Seralabs) and cloned into microtiter plates by single cell picking following mitotic shake off from subconfluent cultures. Several 'parental' clones were picked on the basis of flat, 'normal looking' morphology, expanded, and one selected for infection with retrovirus supernatants. Balb/c (1–3) are sister clones, from the same bulk population, which do not express IGF-II from their own endogenous gene. Balb/c (1) is the parent clone used for infection with virus. Infection and cloning of virus carrying lines was carried out essentially as described in Boulter and Wagner, (1988) 5×10^4 Balb/c cells were plated onto 60 mm diameter dishes (Nunc) in Alpha/Ham/10% foetal calf serum and infected with virus bearing cellular supernatant. Clones resistant to $800 \mu\text{g ml}^{-1}$ G418 (GIBCO UK Ltd.) were isolated after 14 days and expanded using a 3T3 regime. BB4, Bal12, Bal9 and Bal4 were all clonal cell lines selected on the basis of productive construct expression.

For growth experiments cells were plated out onto 60 mm diameter Primaria (Falcon, Beckton-Dickinson, New Jersey, USA) dishes in Alpha/Ham/10% foetal calf serum. After attaching overnight they were washed $5 \times$ in serum free medium and overlaid with 5 ml of Alpha/Ham containing

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0.5% foetal calf serum. Cell numbers were then counted daily in triplicate in parallel dishes over the remaining 13 days. IGF-II secretion was measured by plating 5×10^5 cells in 90 mm petri dishes in a 50/50 mixture of Alpha modified Eagles medium/Hams F12 (Alpha/Ham) supplemented with $10 \mu\text{g ml}^{-1}$ transferrin and 10% foetal calf serum (Biddle *et al.*, 1988). Subconfluent cells were washed and overlaid with 5 ml of the same medium as above but lacking foetal calf serum. Medium was collected after 16 h and subjected to extraction and radioimmunoassay according to Hill *et al.* (1989).

Tumourigenicity assays

5×10^6 cells in phosphate buffered saline harvested from subconfluent cultures by treatment with trypsin/versine (Bernstine *et al.*, 1973) were injected subcutaneously over the scapula of nude mice in a volume of 0.1 ml. In all experiments presented, 6 week old Balb/c nu/nu mice were used, five mice per graft. In parallel experiments similar results were obtained using CBA nu/nu ♀ and outbred nu/nu ♀ with five mice per cell line. In a separate experiment (see Table I) 5×10^6 BB4 cells were mixed with 5×10^6 Antisense construct containing cells before inoculation into a single suprascapular site.

Balb/c nu/nu mice were obtained from the MRC Clinical Research Centre, Harrow, others were bred in house. All experiments were carried out according to the Medical Research Council UKCCR guidelines. Mice were fed *ad libitum* during the experiment and palpated daily. 'Latency' represents the time taken from grafting to the first palpable growth of around 1 mm^3 (estimated with calipers) and the experiments continued for 18 months in the case of the single animal which did not show a tumour.

Viral constructs

A human IGF-II cDNA was derived from the hepatoma cell line previously described (Schofield & Tate, 1987). This variant cDNA contained a polymorphism in the B domain of the peptide converting glu-6 to gly-6. The cDNA was cloned into the Bgl II site of the replication defective retroviral vector pXT1 (Boulter & Wagner, 1990) by linker substitution such that the IGF-II cDNA was driven from the HSV TK promoter. Genomic and spliced genomic transcripts (marked g and sg respectively in Figure 1), give rise to the product of the neo gene and the TK driven transcripts to IGF-II protein. Viral genomes were transfected into the packaging line PA317, and G418^r clones selected as viral producers for infection of the ecotropic packaging line Psi-2 (Mann *et al.*, 1983). G418^r clones of psi-2 were then selected for production of retrovirus. The line used produced 0.5×10^5 cfu ml⁻¹, when titred on Balb/c fibroblasts. All cell clones infected with IGF-II carrying retrovirus used in the grafting experiments were found to be free of helper virus.

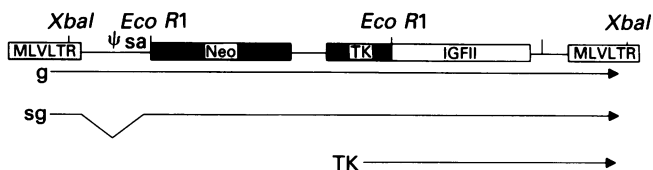


Figure 1 A human IGF-II cDNA was derived from the hepatoma cell line HEP-G2 as described in Materials and methods and cloned into the Bgl-II site of the replication defective vector pXT1 by linker substitution such that the IGF-II cDNA was driven from the HSV TK promoter. Genomic and spliced genomic transcripts (marked g and sg respectively), give rise to the product of the neo^r gene, and the TK driven transcripts to IGF-II protein.

Expression of retroviral constructs in Balb/c fibroblasts

5×10^4 Balb/c cells were plated onto 60 mm dishes in Alpha/Ham/10% foetal calf serum and infected with virus bearing cellular supernatant as described previously. Clones resistant to $800 \mu\text{g ml}^{-1}$ G418 were isolated after 14 days and expanded. Polyadenylated RNA was prepared from subconfluent cultures, electrophoresed on denaturing formaldehyde gels and blotted to nitrocellulose.

Nucleic acid analysis

Polyadenylated RNA was prepared from subconfluent cultures by treatment with protease K and batch adsorption onto oligo dT cellulose (Pharmacia) according to Boulter & Wagner (1988), electrophoresed on denaturing formaldehyde gels and blotted to nitrocellulose (Lehrach *et al.*, 1977). Filters were hybridised to an IGF-II probe derived from the parent construct. Markers were an RNA ladder purchased from BRL Ltd, and positions shown above. Provirus copy number was estimated by digestion of genomic DNA from clones with XhoI and subsequently hybridisation with the neo^r gene probe (Coulombe & Skup, 1986). Control hybridisation was carried out using a mouse alpha-globin probe kindly provided by Dr Emma Whitelaw (Whitelaw *et al.*, 1989).

Results

Effects of expression of IGF-II in vitro

A human IGF-II cDNA was isolated from a hepatoma cell line, Hep G2, and cloned into the BglII site of the retroviral vector pXT1, as described in Materials and methods. Viral genomes were packaged in the ecotropic packaging cell line psi-2 and used to infect a cloned subline of Balb/c/3T3 fibroblasts (Balb/c (1)). G418 resistant colonies resulting were cloned and characterised, and four clones were chosen which expressed the introduced gene at different levels. Clones bearing a construct with the IGF-II gene inserted 3'-5' with respect to the HSV Thymidine Kinase (TK) promoter were similarly obtained and were designated 'antisense' constructs (see legend to Figure 1). Levels of mRNA from the internal

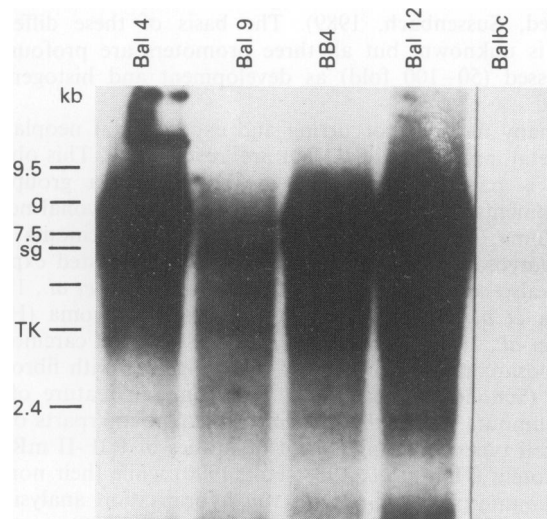


Figure 2 Expression of retroviral constructs in Balb/c clones. Filters were hybridised to an IGF-II probe derived from the parent construct. Markers were an RNA ladder purchased from BRL Ltd, and positions shown above. BB4, Bal12, Bal9 and Bal4 were all clonal cell lines selected on the basis of productive construct expression. Balb/c is the parent clone, Balb/c (1), which does not express IGF-II from its own endogenous gene. Genomic, spliced genomic and transcripts originating from the TK promoter are marked as g, sg and TK respectively.

TK promotor, which are the only species capable of translation into IGF-II peptide, were generally low in comparison with the LTR derived genomic transcripts (see Figure 2), but radioimmunoassay showed that the cells with the 'sense' constructs secreted IGF-II into tissue culture supernatant (Table I). No correlation was seen between the morphology of individual clones and the levels of secreted protein.

The effect of expression of IGF-II on cell growth in culture was assayed by transfer to 0.5% serum containing medium, and by counting cell numbers over the next 14 days (see Figure 3). When placed in 0.5% serum, the cell lines express-

ing IGF-II at levels $> 6 \text{ ng}/10^6 \text{ cells}/24 \text{ h}$ assumed a more flattened aspect, whereas the control cell lines were much more refractile and poorly attached. This difference was only seen on serum starvation. Growth of control cells over the first 8 days was slower than those making IGF-II, and the control cells reached a much lower density after 14 days: $1.17 \times 10^4 \text{ cm}^{-2}$ as opposed to $5 \times 10^4 \text{ cm}^{-2}$ for the most rapidly growing. Supplementation of the medium of control cells with 10 ng ml^{-1} of human recombinant IGF-II (Eli Lilly) daily showed a marked recovery in the rate of population growth similar to that obtained by autocrine expression (Figure 3a). We conclude that the expression of IGF-II by clones of the Balb/c/3T3 cell line aids their growth under conditions of serum starvation.

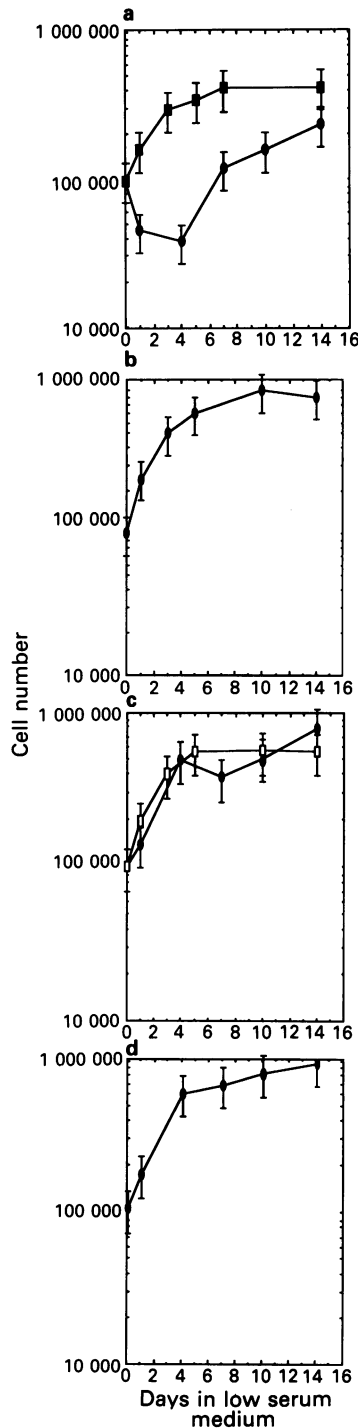


Figure 3 Effects of expression of IGF-II on growth of infected clones and controls under conditions of serum starvation. a, ●, Balb/c (1), control cell line, ■, Balb/c supplemented with 10 ng ml^{-1} recombinant human IGF-II daily. b, Bal12, c, □, Bal9, ●, Bal4. d, BB4. Cell numbers were counted daily in triplicate in parallel dishes over the remaining 13 days. Each point is the result of four replicates for each experiment.

Effects of IGF-II expression on growth of grafts in nude mice

Grafting of four experimental cell lines, and four control lines (the parental clone (Balb/c (1), two sister clones (Balb/c (2) and (3)) derived at the same time but not infected with the virus, and an antisense construct containing clone) showed unexpectedly, that growth of cells bearing IGF-II constructs in nude mice was dramatically inhibited. (Table I). Cells (5×10^6) were inoculated subcutaneously over the scapula of Balb/c nu/nu mice. The site of injection was palpated daily and the latency of tumour formation estimated by the time taken for a noticeable nodule to form (generally about 1 mm in diameter). Thereafter tumour size was estimated by caliper measurements and the experiments terminated when the tumour reached a weight of approximately 1 g. The longest mean latency observed was 76.2 days. Once tumours were detected, the average increase in weight from first palpation to harvest was almost identical for all groups at $20 \text{ mg day}^{-1} \pm 0.04 \text{ mg (s.d.)}$. Tumour histology at 2 weeks showed small nests of undifferentiated cells, while primitive fibrosarcomas dominated the tumours at the end of the experiment. Little difference between the cell lines was apparent, either in terms of vascularisation or differentiation, but tumours derived from cells which originally expressed IGF-II did show approximately 50% more mitotic figures per field than controls when examined at the end of the experiment.

We attempted to establish cell lines from tumours, both in the presence and absence of $800 \mu\text{g ml}^{-1}$ G418, and then analysed DNA and RNA from them. Surprisingly G418 resistant cell lines were difficult to obtain from experimental tumours whose progenitor cells were G418^r. Analysis of DNA and RNA from both primary tumours and cultures established without G418 present indicated that in the major-

Table I Effects of IGF-II expression on tumour formation in nude mice

Graft	Intact provirus copy number	IGF-II secreted/ 10^6 cells/24 h	Latency (days \pm s.d.)	Tumour take incidence
Balb/c (1)	0	0	14.75 ± 3.8	8/8
Balb/c (2)	0	0	17.5 ± 2.3	5/5
Balb/c (3)	0	0	12.5 ± 1.2	5/5
Bal9	1	5.9	14.7 ± 4.2	10/10
Bal4	1	8.2	61.7 ± 5.0	10/10
BB4	1	11.0	76.2 ± 10.0	10/10
Bal12	1	12.7	54.1 ± 3.8	9/10
Antisense	1	0	12.0 ± 2.0	10/10
BB4/Antisense ^a	1/0	11.0/0	16.1 ± 3.1	5/5

Balb/c (1) represents the parental cloned cell line, Balb/c (2), and Balb/c (3) cell lines cloned from the same population as Balb/c (1) but not used for expression. Bal9, Bal4, Bal12 and BB4 individual clones infected with IGF-II expressing retroviruses, and Antisense, a clone carrying a retrovirus with the IGF-II cDNA in reverse orientation with respect to the TK promoter. BB4/Antisense^a represents the data from an experiment in which 5×10^6 BB4 cells were mixed with 5×10^6 Antisense construct containing cells before inoculation into a single site. Groups of five mice were used for each experimental set as described in Materials and methods.

ity of cases (35/40) the tumour cells had lost the active introduced copies of the virus (see Figure 4). Of the remaining five cases the IGF-II sequences were truncated in one instance, did not produce mRNA transcripts (e.g. XG7 in Figure 4), presumably due to point mutations, in two cases, since no change in the restriction map was evident; and unaffected in the two remaining tumours. From these results it is clear that Bal 9 is unusual in that it loses the IGF-II sequences very rapidly (see Table I). Karyotype analysis was conducted on cell lines established from tumours in the absence of G418 selection, and demonstrated no consistent pattern of chromosome loss or gain during the latent period.

IGF-II is usually active as a secreted peptide and we wished to see if it were possible to obtain a *trans*-acting suppression of tumour formation by mixing equal numbers of an IGF-II expressing clone (BB4) with the antisense containing line. 5×10^6 cells of each line were inoculated suprascapularly into a single site. The results are shown in Table I, and indicate that no obvious *trans* acting effect could be obtained.

Discussion

It has previously been reported that Balb/c/3T3 fibroblasts require insulin-like growth factors to proliferate under conditions of serum starvation (Wharton *et al.*, 1981). This de-

pendence is not only reflected in their rate of proliferation but also in their ability to grow in an anchorage independent fashion (Massague *et al.*, 1985). We confirm these findings and have demonstrated that this requirement may be abrogated by provision of endogenously synthesised peptide. Addition of exogenous recombinant IGF-II to non-expressing cells stimulated cell proliferation markedly but seemed to be less effective than autocrine expression in sister clones in terms of nominal available concentration/cell when compared with the measured rates of secretion of IGF into the medium. This may be explained by continuous internalisation of autocrine IGF-II during growth, leading to an underestimate of the rate of production.

The three parental clones from our original population of target cells were of similar morphology, and little variability was observed in their ability to produce tumours in nude mice. This is broadly in agreement with previous studies (Rubin, 1988) though less clonal variability in tumourigenicity was seen in the experiments described here. Surprisingly, whilst an autocrine growth loop was apparently operating in the cells *in vitro*, expression of IGF-II acted to suppress tumour formation *in vivo*. Loss of the introduced construct in the majority of the experiments suggests that the expression of IGF-II is strongly selected against during tumour formation in immunodeprived mice and by this criterion IGF-II is a tumour suppressor gene. The suppressive effect *in vivo* probably relies on an interaction between graft and host, because expression acts to promote cell growth *in vitro* (Figure 3). We consider it unlikely that there was extensive host contribution to the tumours, because the relative copy number of viral sequences in the tumours derived from antisense constructs was always commensurate with that seen in the inoculated cells. The karyotypes of cell lines derived from the tumours were clearly not of host origin, and resembled those of the injected cells, without any general pattern of chromosome loss. These results are consistent with selection directed against cells expressing IGF-II constructs, since cells with the antisense construct gave rise to tumours within 14 days of grafting (10/10); all these tumours retained the construct and, when explanted, readily gave rise to G418^r cultures. A similar pattern of construct loss from grafts carrying presumptive tumour suppressor genes has also been reported for the IL4 gene (Tepper *et al.*, 1988). However, in contrast to the systemic effects of IL4 on tumour suppression implantation of expressing and non-expressing clones together did not result in a *trans* effect. This suggests a cell autonomous process such as that described by Copeman and Harris (1990).

Identification of IGF-II as a tumour suppressor in this system is surprising because in the clinical situation levels of IGF-II mRNA are often much higher in tumours than in the surrounding adult host tissue in which they are found (Scott *et al.*, 1985; Su *et al.*, 1989; Cariani *et al.*, 1988; Hoppener *et al.*, 1988; Daughaday *et al.*, 1988; Tricoli *et al.*, 1986). In general only the presence of mRNA has been examined and few experiments have addressed the concurrent presence of IGF-II peptide. This paradox may therefore be explained by the operation of various mechanisms both acting to reduce gene dosage and to regulate the production of bioactive IGF-II post transcriptionally. The results presented here suggest that in some types of tumour the IGF-II genes should be either reduced in dosage or altered in some way to produce an IGF-II protein which does not promote tumour suppression.

In sporadic Wilms' tumour (Reeve *et al.*, 1989), hereditary Wilms; tumour, Beckwith-Weidemann syndrome (Ping *et al.*, 1989), adrenocortical carcinoma, rhabdomyosarcoma (Koufos *et al.*, 1985) and mammary carcinoma (Ali *et al.*, 1987) allele loss at 11p15.5 is frequently detected with allele specific polymorphisms in the insulin gene, and these losses are not always associated with detectable changes in the more proximal region of the short arm. As the insulin gene is only 1.4 kb 5' of the IGF-II locus, loss of an insulin allele is almost certainly accompanied by loss of the adjacent IGF-II locus. A reduction in IGF-II gene dosage or rearrangement

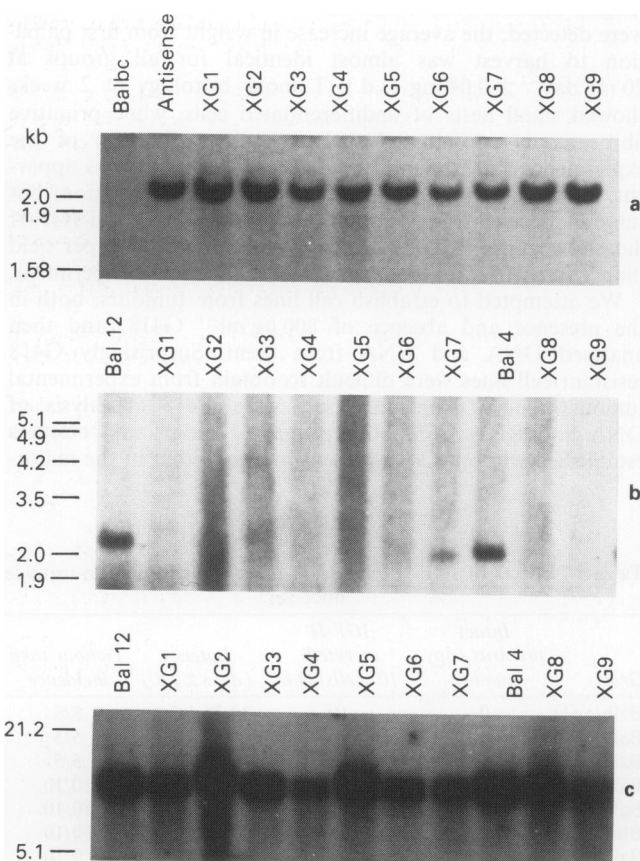


Figure 4 Pattern of construct retention and loss from three representative sets of allograft tumours derived from retrovirally infected cells lines. **a**, Balb/c; parental cell line, Antisense; clonal cell line containing proviral construct with IGF-II cDNA in antisense orientation with respect to TK promoter. XG1-XG9 are allograft tumour DNAs derived from the antisense clone. **b**, Bal12; clonal cell line containing IGF-II expressing proviral construct, XG1-XG-7, allograft tumours derived from Bal12 after extended latency. Bal4; clonal cell line containing IGF-II expressing proviral construct. XG8-XG9; representative allografts derived from Bal4; **c** filter **b** reprobed with the mouse a-globulin gene probe derived from MpSVod. (28). The markers used were an *HindIII/EcoRI* digest of bacteriophage lambda.

has also been found in such tumours (e.g. Irminger *et al.*, 1989) which would not be expected if selection were occurring in the tumour for autocrine growth based on high levels of expression of bioactive IGF-II. Our results suggest that it may be selective pressure for loss of the IGF-II gene at 11p15 which fully or partially accounts for loss of this region of chromosome 11 in naturally occurring tumours. A similar result may be achieved by translational regulation, rather than genetic loss or inactivation, and inhibition of translation of IGF-II mRNA has recently been shown in several Wilms' tumours (Haselbacher *et al.*, 1987).

We propose that expression of IGF-II mRNA by developmental tumours may be a tumour epiphenomenon, as the postulated progenitor cell types, (e.g. metanephric blastema in the case of Wilms' tumour), are known to make IGF-II mRNA and protein at high levels. Selection against such expression in a cell already transformed might be expected if IGF-II has the same suppressive effect on tumour formation in man as in an experimental situation. In support of this Little *et al.* (1987) using xenografts of primary Wilms' tumours, were able to demonstrate that IGF-II expression was selected against during tumour passage, and they suggested that elevated IGF-II mRNA was not an essential

component of tumour progression. Additionally, Maitland *et al.* (1989) have reported the selective loss of chromosome 11 during maintenance of cell hybrids between normal human foetal kidney and HeLa cells as grafts in nude mice. The reported occurrence of deletions in 11p13 and 11p15 in Wilms' tumours associated with aniridia (WAGR) (Henry *et al.*, 1989) suggests that suppression of IGF-II expression may be important in tumour progression rather than in initiation, the latter perhaps being the function of the 11p13 deletion. Candidate genes in this region have recently been described (Gessler *et al.*, 1990; Rose *et al.*, 1990). Current experiments are being directed towards the mechanism responsible for the tumour suppressing effect of IGF-II.

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