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A transition in transcriptional activation by the glucocorticoid and retinoic acid receptors at the tumor stage of dermal fibrosarcoma development

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In transgenic mice harboring the bovine papillomavirus genome, fibrosarcomas arise along an experimentally accessible pathway in which normal dermal fibroblasts progress through two pre-neoplastic stages, mild and aggressive fibromatosis, followed by a final transition to the tumor stage. We found that the glucocorticoid receptor (GR) displays only modest transcriptional regulatory activity in cells derived from the three non-tumor stages, whereas it is highly active in fibrosarcoma cells. Upon inoculation into mice, the aggressive fibromatosis cells progress to tumor cells that have high GR activity; thus, the increased transcriptional regulatory activity of GR correlates with the cellular transition to the tumor stage. The intracellular levels of GR, as well as its hormone-dependent nuclear translocation and specific DNA binding activities, are unaltered throughout the progression. Strikingly, the low GR activity observed in the pre-neoplastic stages cannot be overcome by exogenous GR introduced by co-transfection. Moreover, comparisons of primary embryo fibroblasts and their transformed derivatives revealed a similar pattern—modest GR activity, unresponsive to overexpressed GR protein, in the normal cells was strongly increased in the transformed cells. Likewise, the retinoic acid receptor (RAR) displayed similar differential activity in the fibrosarcoma pathway. Thus, the oncogenic transformation of fibroblasts, and likely other cell types, is accompanied by a striking increase in the activities of transcriptional regulators such as GR and RAR. We suggest that normal primary cells have a heretofore unrecognized capability to limit the magnitude of induction of gene expression.

Key words: fibrosarcoma/glucocorticoid receptor/retinoic acid receptor/tumorigenesis

Introduction

In general, molecular analyses of mammalian cell functions, such as gene transcription and its regulation, have been carried out with cells that have suffered neoplastic transformation or have been otherwise converted to continuously dividing, established cell lines. Although much valuable information has been extracted from these experimentally tractable models, it is also clear that normal and immortalized cells differ in important ways. Indeed, defining and understanding these differences at the molecular level is critical to our understanding of normal cell function, and of cancer and other proliferative diseases.

The conversion of a normal cell into a neoplastic one appears to occur in multiple steps (Vogelstein and Kinzler, 1993). One approach to studying this process employs transgenic mice carrying dominant negative oncogenes or inactivated tumor suppressor genes (Hanahan, 1988). For example, a small proportion of dermal fibroblasts in mice bearing transgenic bovine papillomavirus type I (BPV-1) genomes proceeds through two histological grades of hyperplasia, termed mild and aggressive fibromatosis, and finally emerges as dermal fibrosarcomas. Cells cultured from each of these stages appear to retain characteristics of the lesions from which they were derived (Sippola-Thiele *et al.*, 1989). All three pathological stages contain BPV-1 DNA and RNA transcripts, and the aggressive fibromatosis and fibrosarcoma cells form tumors after inoculation into mice. Importantly, the aggressive fibromatosis and fibrosarcoma cultures contain similar levels of the BPV-1 E5 and E6 oncogene products (Sippola-Thiele *et al.*, 1989). Thus, the BPV-1 transgene is not a sufficient determinant of the dermal fibrosarcoma phenotype, suggesting that changes in cellular components must contribute to the conversion of the advanced hyperplasia into fibrosarcoma.

The fibrosarcomas contain one or both of two karyotypic defects, neither of which is seen in the fibromatosis stages: translocations involving chromosome 14 (60%), or duplications of chromosome 8 (70%); ~30% of the tumors carry both lesions (Lindgren *et al.*, 1989). It is apparent that sites of consistent chromosome rearrangements can localize genes that may be critically involved in malignant transformation, and that the rearrangements themselves can subvert the normal functioning of these genes (Bishop, 1991; Marshall, 1991; Solomon *et al.*, 1991). In this regard, it seemed potentially interesting that a proto-oncogene, JunB, was found to reside within a region of chromosome 8 (Mattei *et al.*, 1990) that is most commonly duplicated in the fibrosarcomas (Lindgren *et al.*, 1989). A survey of several members of the AP-1 factor family (Vogt and Bos, 1990; Hunter, 1991; Kerppola and Curran, 1991) during the progression to fibrosarcoma revealed that JunB and c-Jun were elevated in the aggressive fibromatosis as well as the fibrosarcoma cultures, whereas JunD and c-Fos remained constant (Bossy-Wetzel *et al.*, 1992). However, overexpression of JunB and/or c-Jun was not sufficient to induce the complete tumor cell phenotype; mild fibromatosis cells overexpressing either or both of these genes displayed anchorage-independent growth in soft agar, but failed to produce tumors upon inoculation into histocompatible mice. Thus, it appears that additional events during tumor progression distinguish aggressive fibromatoses from fibrosarcomas.

Extensive interactions have been described between AP-1 factors and various members of the 'intracellular receptor' superfamily, including the estrogen receptor (ER) (Gaub *et al.*, 1990; Doucas *et al.*, 1991), the retinoic acid (RAR) and vitamin D (VDR) receptors (Schüle *et al.* 1990a), and especially the glucocorticoid receptor (GR) (Diamond *et al.*, 1990; Jonat *et al.*, 1990; Schüle *et al.*, 1990b; Yang *et al.*, 1990). The intracellular receptors mediate the effects of various small ligands, including steroids, thyroid hormone, retinoids and vitamin D (for reviews see Evans, 1988; Burnstein and Cidloswki, 1989; Ham and Parker, 1989; Truss and Beato, 1993). Although distinct in detail, these receptors share general characteristics of structure and mode of action. Thus, the GR binds its cognate hormone in the cytoplasm, migrates to the nucleus and regulates transcription upon association with specific glucocorticoid response element (GRE) DNA sequences near target genes. Two broad classes of GREs have been described: GR binding at 'simple GREs', which contain imperfect palindromes of hexamer half-sites separated by 3 bp (Beato, 1989), is sufficient for enhancement of transcription from nearby promoters, although repression has not been observed from such sites. In contrast, GR acts only in collaboration with other factors that bind at 'composite GRE' sites that lack a common consensus sequence; at these sites, GR can either enhance or repress transcription (Yamamoto *et al.*, 1992).

Steroid hormones are essential regulators of normal cell growth, differentiation and homeostasis (for review see Walsh and Avashia, 1992). For example, estrogens and androgens can function as powerful mitogens while also playing critical roles in differentiation (Wilding, 1992; Jordan and Morrow, 1993), whereas glucocorticoids tend to promote differentiation and inhibit proliferation. Steroids are also commonly used therapeutics. Thus, topical glucocorticoids reduce tissue destruction in certain skin disorders by down-regulating type IV collagenase (Oikarinen *et al.*, 1993). Intracellular receptors and their ligands have been also implicated in various malignancies. For example, the RARβ gene can be rearranged as a result of hepatitis B virus integration in certain human hepatocellular carcinomas (Dejean *et al.*, 1986), and RARα is split into two chimeric proteins by a t(15:17) translocation in acute promyelocytic leukemia (Borrow *et al.*, 1990; De Thé *et al.*, 1990). Estrogens appear to promote the course of several cancers (Jordan and Murphy, 1990). Glucocorticoids inhibit the growth of carcinogen-induced tumors in mouse lung (Droms *et al.*, 1993), mouse skin (Strawhecker and Pelling, 1992) and rat colon (Denis *et al.*, 1992). In contrast, glucocorticoids markedly enhance the transformation of cultured human epithelial cells by Kirsten murine sarcoma virus (Durst *et al.*, 1989), and strongly activate mouse mammary tumor virus gene transcription and virus production (Truss and Beato, 1993).

The functional interactions of intracellular receptors with AP-1, together with the established effects of steroids and retinoids on cell growth and differentiation, raised the possibility that certain activities of an intracellular receptor might differ in normal and immortalized cells in general, or might even contribute to the progression to fibrosarcoma. As a first step toward examining these broad possibilities, we assessed the expression and activities of GR and RAR during the multistep tumorigenesis process, and in other normal and transformed cells.

Results

GR activity is low before the fibrosarcoma stage

To monitor the activity of endogenous GR during dermal fibroblast tumorigenesis, we performed transient transfection assays using low-passage primary cell lines representative of the four stages in this multistep pathway: normal dermal fibroblasts, NF; mild fibromatosis, MF; aggressive fibromatosis, AF; fibrosarcoma, FS. We tested the transcriptional activity of various GREs, positioned upstream of a minimal promoter and fused to luciferase reporter sequences (de Wet *et al.*, 1987); the results were normalized to a co-transfected β-galactosidase expression vector. We first measured the hormone responsiveness of a reporter construct, TAT3-Luc, containing three tandem simple GREs derived from the tyrosine aminotransferase (TAT) gene (Jantzen *et al.*, 1987). Surprisingly, dexamethasone, a synthetic glucocorticoid, evoked only a modest stimulation of the low basal luciferase activity in the NF, MF or AF lines, whereas strong hormonal induction was observed in the FS cells (Figure 1A); similar results were obtained using a reporter bearing a different simple GRE (Sakai *et al.*, 1988) derived from the mammary tumor virus LTR (data not shown). Thus, we conclude that strong endogenous GR activity is observed in the final FS stage cells, but not in the initial three stages of the pathway.

Bossy-Wetzel *et al.* (1992) detected substantially more AP-1 DNA binding activity in extracts of the AF and FS cells than in NF or MF cell extracts. To extend these findings, we next tested the transcriptional activity of endogenous AP-1 factors using a luciferase reporter construct bearing an AP-1 response element from the collagenase promoter (−73)Col-Luc (Angel *et al.*, 1987). Indeed, we found that AP-1 activity was somewhat greater in the AF and FS cells relative to the NF and MF cells from the initial stages (Figure 1B). Thus, both GR and AP-1 activities increase in the course of tumor progression, but the stages at which the factors become activated differ.

In view of the observed changes in GR and AP-1 activities, we next tested a composite element that requires both factors for transcriptional activation. The synthetic composite element, G(26)A, which resembles a sequence in the glucocorticoid regulatory region of the glutamine synthetase gene (Zhang and Young, 1991), contains the TAT GRE sequence linked through a 15 bp spacer to the collagenase AP-1 sequence (W.Matsui and K.R.Yamamoto, unpublished data). In this configuration, neither GR alone nor AP-1 alone (either Jun homodimers or Jun−Fos heterodimers) activates a linked reporter gene in transfected embryonal carcinoma F9 cells (which express low levels of endogenous GR and AP-1 factors), whereas co-transfection of GR and AP-1 (Jun−Jun or Jun−Fos) produces a strong synergistic activation upon hormone addition (W.Matsui and K.R.Yamamoto, unpublished data). We transfected a luciferase reporter construct containing three copies of the G(26)A composite element, G(26)A3-Luc, into cell cultures representing each stage of the fibrosarcoma progression. As expected, luciferase activity was dramatically induced by dexamethasone in the tumor cells (Figure 1C), supporting the view that GR

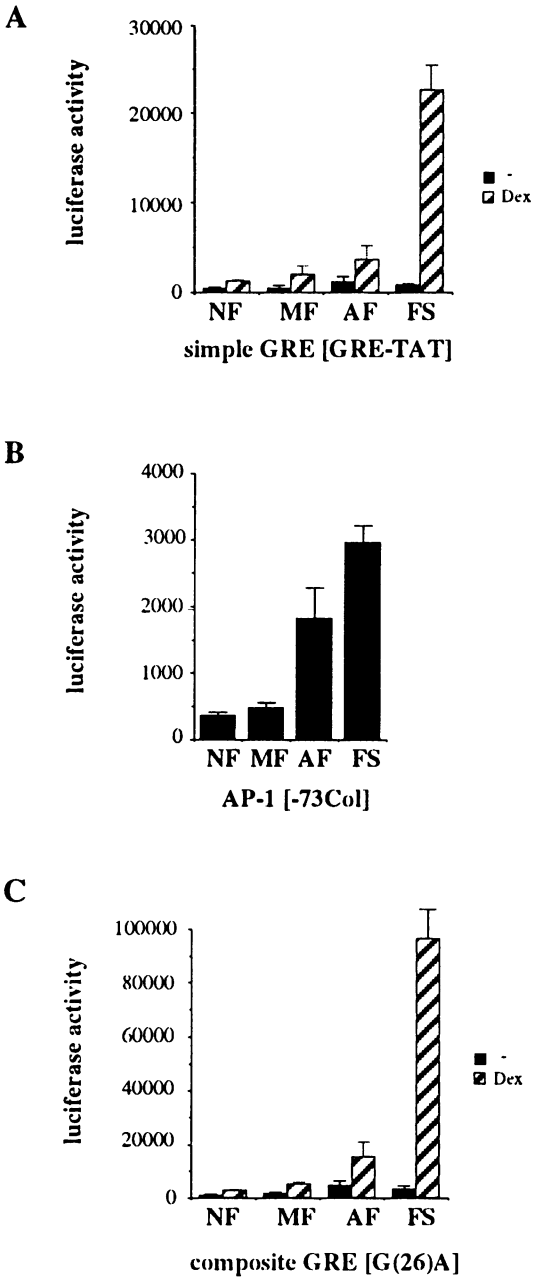

Fig. 1. Endogenous GR activity during fibrosarcoma progression. (**A**) Expression of the TAT3-Luc reporter construct in normal dermal fibroblasts (NF 40950), mild fibromatosis (MF 14249), aggressive fibromatosis (AF BPV3) and fibrosarcoma cells (FS BPV1). The cells were either untreated (black bars) or treated for 24 h with 100 nM dexamethasone (hatched bars). Transcriptional activity of endogenous receptor was analyzed. In all transfection experiments, the luciferase activity was normalized to the β-galactosidase activity of a co-transfected reporter. The values presented are the average of at least six different experiments. (**B**) Expression of the (−73)Col-Luc reporter construct during fibrosarcoma development (NF, MF, AF and FS cells, as in A). The activity of endogenous factors present in these cells was assayed without hormone treatment. The same qualitative results were obtained using three copies of the consensus AP-1 binding site from the collagenase promoter, located upstream of the minimal alcohol dehydrogenase promoter (data not shown). (**C**) Expression of the G(26)A3-Luc reporter construct in NF, MF, AF and FS cells in the absence (solid bars) or presence of dexamethasone (hatched bars). Conditions as in (A).

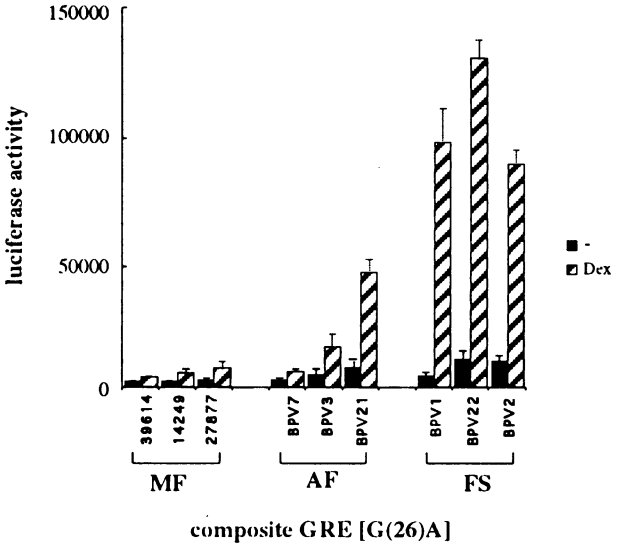

Fig. 2. GR transcriptional regulatory activity among different cell lines from mild fibromatosis, aggressive fibromatosis and fibrosarcoma. The G(26)A3-Luc reporter was transfected into different cell lines isolated from MF (39614, 14249, 27877), AF (BPV7, BPV3, BPV21) and FS (BPV1, BPV22, BPV2). The cells were untreated (solid bars) or treated with 100 nM dexamethasone (hatched bars). Luciferase activity was normalized against a co-transfected β-galactosidase reporter.

and AP-1 are both highly active at this stage. However, despite the presence of elevated AP-1 activity in the AF cells (Bossy-Wetzel *et al.*, 1992; see also Figure 7), only a modest hormonal induction was conferred through the G(26)A element at this stage.

In contrast to the observed changes in GR and AP-1 activities in these cell cultures, overall transcription levels did not change; the Rous sarcoma virus (RSV) promoter, for example, displayed similar activity in cell lines from all four stages (data not shown). Similarly, the regulatory factor SP1 activated transcription from the thymidine kinase promoter (−109) to similar levels in all cell lines. We conclude that GR undergoes a selective and striking increase in its capacity to activate transcription, both through simple and composite GREs, at the final stage of fibrosarcoma formation.

GR activity in the AF stage correlates with tumorigenic potency

Our initial analysis involved representative cell cultures from the four identified stages of the tumorigenesis pathway (NF 40950, MF 14249, AF BPV3, FS BPV1). To assess the extent of variation among different lines from a given stage, we assayed the activity of GR on the G(26)A composite element in three or more independent cell lines from each stage. We found that all lines derived from normal dermis and mild fibromatoses displayed low GR activity, whereas GR function in all of the fibrosarcoma clones examined was consistently strong (Figure 2). In contrast, aggressive fibromatosis cultures spanned a range of GR activity (Figure 2). Thus, AF BPV7 carried no more GR activity than the MF lines, AF BPV21 contained ~40% of the activity found in a robust FS line, and AF BPV3 displayed an intermediate phenotype.

The observed differences in GR activity among aggressive fibromatosis clones allowed us to test whether GR

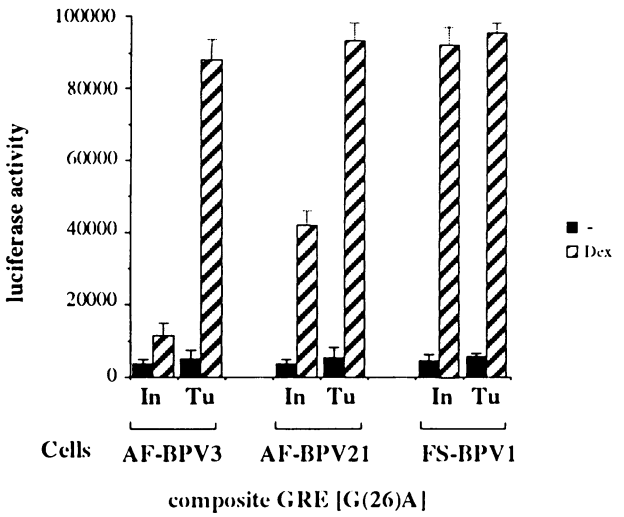

Fig. 3. GR transcriptional regulatory activity in tumors derived from inoculation of aggressive fibromatosis and fibrosarcoma cells. Expression of the G(26)A3-Luc reporter is shown for AF (BPV3 and BPV21) and FS BPV1 cells before inoculation (In) into nude mice, and in tumor cells (Tu) derived from those inoculated cells 24 days later. The cells were untreated (solid bars) or treated with 100 nM dexamethasone for 24 h (hatched bars).

activity and 'tumorigenic strength' might be related. Therefore, we inoculated 10^4 (or 5×10^5) cells from each AF cell line and from one representative FS line (BPV1) subcutaneously into nude mice, and monitored formation of tumors. We found that the rate of formation and the relative mass of the tumors were in a rank order: FS-BPV1> AF-BPV21≥ AF-BPV3> AF-BPV7 (data not shown). Therefore, the differences in GR activity during tumor progression roughly paralleled oncogenic potential.

GR is increased in tumors derived from aggressive fibromatosis cells

A prediction of the apparent correlation of GR activity with tumor potential and growth is that GR activity should be high in tumors that arise in mice as a result of progression of inoculated AF stage cells, even if the inoculated cells originally carried only low GR activity. We tested for such a switch in GR function by inoculation into nude mice of AF-BPV3 or AF-BPV21 cells, which have low and intermediate GR activity, respectively; we then assayed dexamethasone responsiveness of the G(26)A composite element in cells from the resultant tumors. As shown in Figure 3, all the tumor-derived cell lines displayed high GR activity; similar results were obtained with the simple TAT GRE (data not shown). Specifically, the AF-BPV3 and AF-BPV21 cells appeared to undergo specific transitions in GR activity, each resulting in tumor cells displaying GR activities comparable with those measured in the bona fide FS-BPV1. As a control, we showed that the GR activity of FS-BPV1 itself was unaffected by the inoculation and re-derivation procedure (Figure 3). These results indicate that a switch in GR activity occurred *in vivo* that correlates with the tumor cell transition.

GR protein is expressed at similar levels throughout progression

One mechanism of GR up-regulation would involve increased transcription of the GR gene. To address this

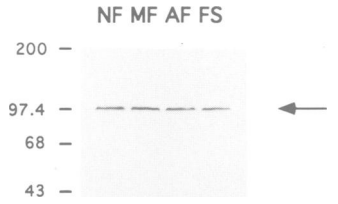

Fig. 4. Intracellular levels of GR protein during fibrosarcoma progression. Immunoblot analysis of endogenous GR from NF 40950, MF 14249, AF BPV3 and FS BPV1. Cell extracts from NF, MF, AF and FS cells were probed with a monoclonal antibody (BUGR2) directed against the DNA-binding domain of GR. Antibody–protein complexes were visualized by enhanced chemiluminescence (ECL, Amersham). Molecular weight (kDa) standards are indicated at left; arrow indicates GR position.

possibility, we analyzed GR production and accumulation by immunoblotting of cell extracts from each of the four stages of tumorigenesis. We found that GR protein accumulated to similar levels in NF, MF, AF and FS cells (Figure 4). Thus, the striking increase in GR activity at the fibrosarcoma stage is not due simply to increased production of GR protein. This result suggests either that the receptor itself somehow differs when produced in fibrosarcoma cells relative to cells from the prior stages, or that the receptor interacts with a non-receptor component that modulates GR activity differentially in early stage cells and fibrosarcoma.

Transfected GR fails to affect GR activities characteristic of each stage

To test for factors that might suppress GR function in the three initial cell types, we co-transfected a GR expression vector together with a simple GRE reporter, TAT3-Luc, into cell lines representative of each of the four stages. The results (Figure 5) revealed that exogenously added GR has no effect on the hormone responsiveness of the NF, MF or AF cultures. In the experiment shown, co-transfected GR only slightly increased the magnitude of the dexamethasone response of the FS cells; however, at lower hormone concentrations, we detected increased transcriptional activity with GR co-transfection of the FS cells (data not shown). In contrast, over a wide range of co-transfected GR expression vector (5 ng to 5 μg; 0.5 μg is shown in the figure, as an example), no systematic difference in the overall GR activity was detected in pre-neoplastic stages, except at the highest doses, where a modest reduction in the hormone response was observed (data not shown). To examine the possibility that the BPV transgene was somehow compromising GR function in normal dermal fibroblasts, we isolated and tested dermal fibroblasts from non-transgenic mice (nNF, or non-transgenic dermal fibroblasts, see Figure 5); the same results were obtained, namely a low transcriptional response to dexamethasone and lack of effect of co-transfected GR. In contrast, the same exogenous GR expression vector increased by some 20-fold the level of transcriptional activation by dexamethasone in embryonal carcinoma F9 cells (Figure 5) and also markedly up-regulated GR transcriptional activity in HeLa cells (data not shown). Notably, co-transfected RSV-β-gal produced comparable β-galactosidase levels among F9 cells and the nNF, NF, MF, AF and FS cells, suggesting similar transfection

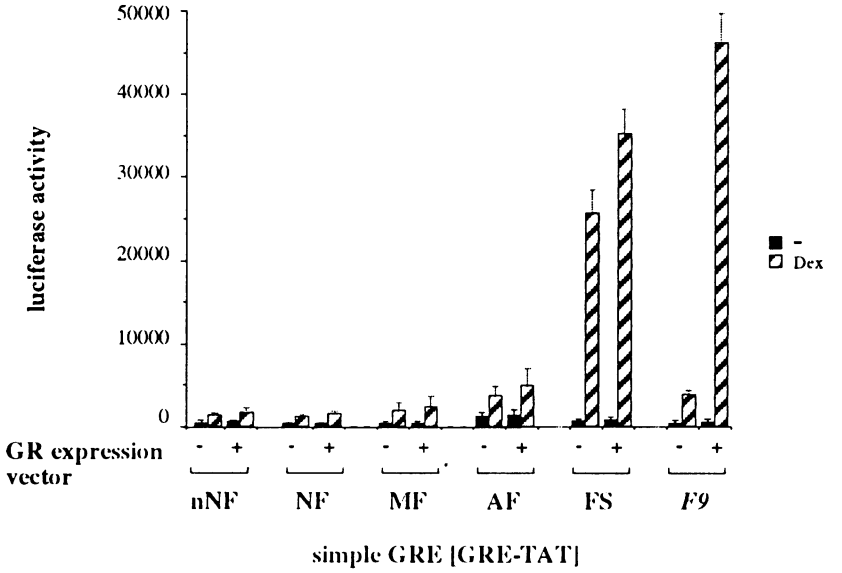

Fig. 5. Effects of co-transfected GR on hormone responsiveness of cell lines from different stages of fibrosarcoma progression. Expression from the TAT3-Luc reporter in NF 40950, MF 14249, AF BPV3 and FS BPV1 cells in the absence or presence of exogenous GR. Cells were co-transfected with the reporter gene and, as indicated, with 6RGR, an GR expression vector. Transfected cells were cultured for 24 h in the absence (solid bars) or presence (hatched bars) of 100 nM dexamethasone. A wide range of 6RGR plasmid (from 5 ng to 5 μg) was tested with similar results; as an example, co-transfection of 0.5 μg 6RGR is shown. As controls, normal dermal fibroblasts from non-transgenic mice (nNF) and embryonal carcinoma F9 cells were also co-transfected with TAT3-Luc, with or without 6RGR, and treated with or without dexamethasone. All cell types displayed comparable levels of β-galactosidase activity (used as endogenous control).

efficiencies. Taken together, these findings imply that the early stage cells suppress GR activity in a manner that cannot be overcome by addition of exogenous GR.

GR undergoes hormone-dependent nuclear translocation in all stages

To begin to assess the nature of the differential GR activity, we first examined the ligand-dependent nuclear translocation of GR in normal dermal fibroblasts and the three progressive conditions, MF, AF and FS. In the absence of ligand, GR in monkey kidney fibroblasts (Picard and Yamamoto, 1987) and rat fibroblasts (Qi *et al.*, 1989) resides predominantly in the cytoplasm, but translocates rapidly to the nucleus upon hormone addition. Consistent with these findings, we found in an immunocytochemical analysis (Figure 6) that GR resides in the cytoplasm in the absence of hormone, and is efficiently translocated to the nucleus upon dexamethasone treatment in all four cell stages. We conclude that competence for nuclear localization of GR is not differentially regulated during tumor progression.

GR binds to DNA in vitro in all stages of tumor development

Another aspect of GR function that might be modulated during tumorigenesis is its recognition and binding of GRE DNA sequences. To examine this possibility, we analyzed by gel mobility shift assays binding to the simple GRE-TAT by GR in nuclear extracts from hormone-treated NF, MF, AF and FS cells. Figure 7A shows that the extracts from the four cell stages were indistinguishable in their GRE binding activities (lanes b, e, h and k). Incubation with 50-fold excess of the unlabeled GRE oligonucleotide (lanes c, f, i and l), or 200-fold excess of non-specific unlabeled oligonucleotide (lanes d, g, j and m) confirmed the specificity of this binding reaction. Moreover, addition of a GR-specific monoclonal antibody to the incubations further retarded the mobility of the DNA–protein complexes (Figure 7A, lanes n-q), demonstrating that GR is present in the complexes.

We also incubated the four nuclear extracts in the presence of a labeled oligonucleotide containing the collagenase AP-1 site, (−73)Col. As previously shown (Bossy-Wetzel *et al.*, 1992), AP-1 DNA binding activity increases during the transition from mild to aggressive fibromatosis cells (Figure 7B). Finally, we tested the DNA binding activity of these nuclear extracts to a labeled oligonucleotide containing a composite response element, G(26)A, which confers transcriptional activation only in the presence of both GR and AP-1 (W.Matsui and K.R.Yamamoto, unpublished results). In gel retardation assays (Figure 7C, lanes b, c, f and i) two major retarded bands were observed (indicated by arrows). We found that binding to the composite element was increased in the AF extracts (compare lanes c and f), consistent with the increased AP-1 activity in those cells. In each case the binding was specific: a 50-fold excess of the unlabeled composite element abolished binding to the labeled probe (lanes d, g and j), whereas a 200-fold excess of a non-specific oligonucleotide had no effect (lanes e, h and k).

We conclude from these experiments that GR can bind *in vitro* to GRE sequences in all cell stages throughout tumorigenesis, and with similar efficiency in the context of a simple GRE, reflecting similar levels of endogenous GR protein. In addition, in the context of a composite element, GR and AP-1 display increased binding activity already at the AF stage, reflecting higher levels of AP-1 protein at that stage. Therefore, differences in DNA binding do not account for the transition in transcriptional activation at the tumor stage.

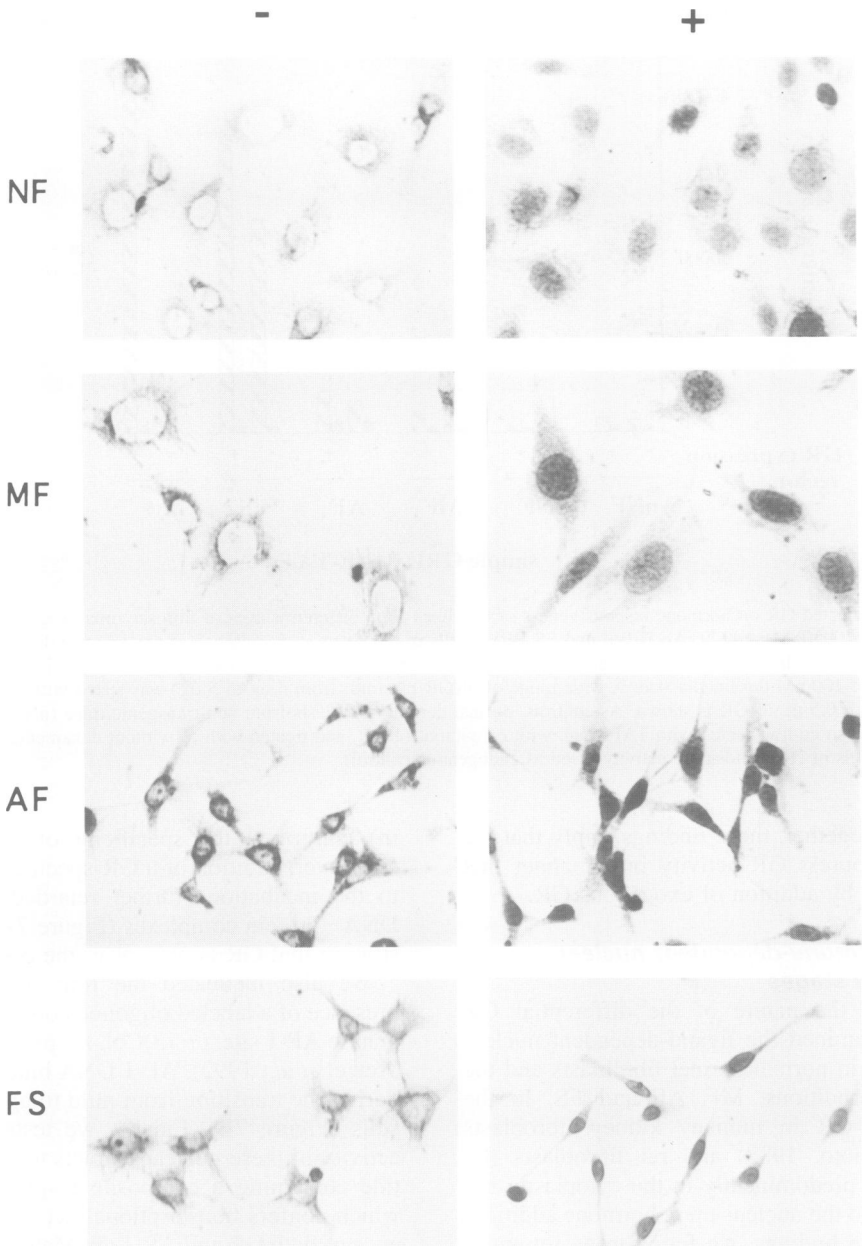

Fig. 6. Nuclear translocation of GR upon hormone binding. NF 40950, MF 14249, AF BPV3 and FS BPV1 cells were cultured in phenol red-free DMEM and either untreated (−) or treated for 2 h with 500 nM dexamethasone (+). Cells were fixed and stained for GR using an immunocytochemical procedure described in Materials and methods. Magnification is 40×. In some, but not all experiments, AF cells seemed to be more intensely stained. Hormone-treated FS cells appeared to be sensitive to our fixation and permeabilization conditions, resulting in an apparent change in their morphology after fixation; this difference was not observed prior to fixation and is not a consequence of hormone treatment *per se*.

Differential activity of GR in normal and transformed cells: a general phenomenon?

The low level of GR activity detected in the normal and pre-neoplastic dermal fibroblasts was unexpected. Interestingly, we had already established that this finding was not simply an artifact of the BPV transgene, as normal dermal fibroblasts from non-transgenic mice behaved similarly (Figure 5). Hence, it seemed conceivable that normal cells might in general carry only modest GR activity that could not be supplemented by exogenously added receptor, and that the robust and supplementable activities typically observed in established cell lines might reflect systematic and potentially important differences between many normal cells and their transformed counterparts.

To begin to examine this possibility, we extended our studies to a different cell type, embryonic fibroblasts. We compared GR activity in primary mouse embryo fibroblasts with that in embryo fibroblasts transformed by *ras* and SV40 large T-antigen. As shown in Figure 8, the magnitude of the hormonal induction of luciferase reporter activity was low in the primary embryo cells relative to their transformed derivatives. In addition, co-transfection of increasing concentrations of a GR expression vector did not increase GR activity in the primary cells, whereas it stimulated significantly the hormone response in the

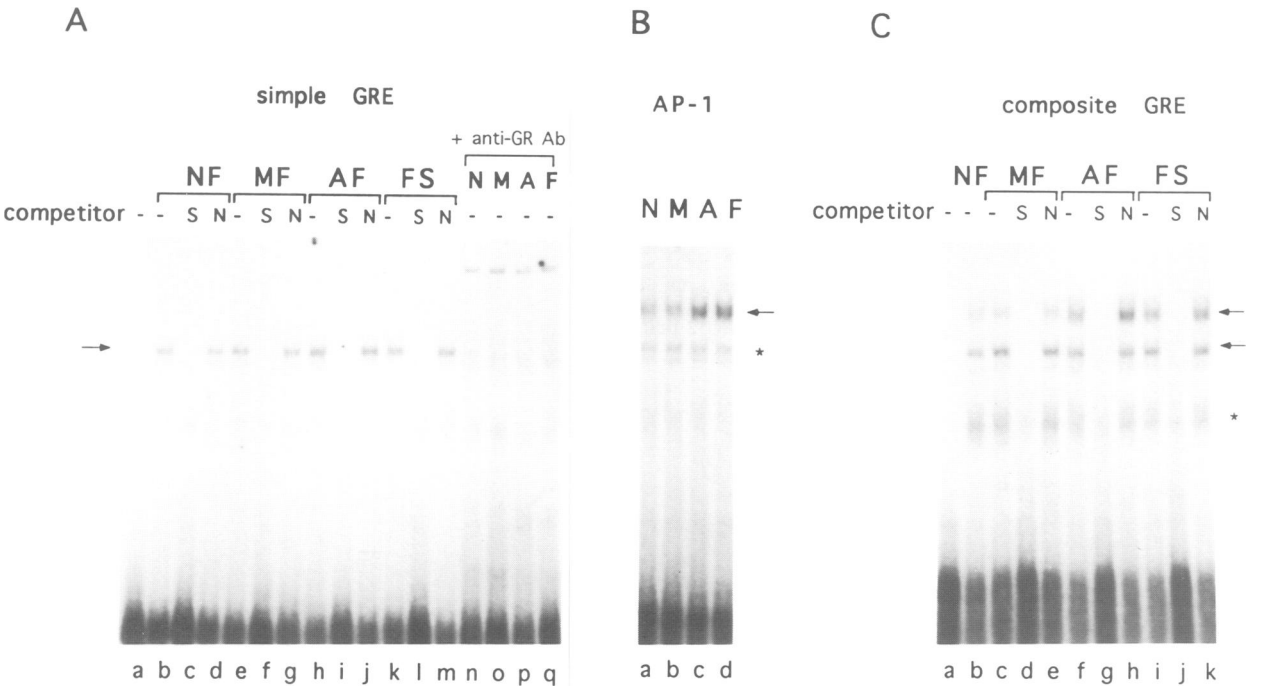

Fig. 7. GRE and AP1 site DNA binding *in vitro* by nuclear extracts from different cell stages during fibrosarcoma progression. (**A**) *In vitro* DNA binding of GR from nuclear extracts of dexamethasone-treated NF, MF, AF and FS cells. An aliquot of the nuclear extracts (5 μg) was incubated in a binding reaction containing 3–5 fmol of a ^{32}P-end-labeled simple GRE-TAT oligonucleotide (see Materials and methods) for 20 min at room temperature (NF, lane b; MF, lane e; AF, lane h; FS, lane k). Protein–DNA complexes were separated by non-denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. Lane a was incubated in the absence of extract. Competition reactions were performed using 50-fold excess of the unlabeled GRE-TAT oligonucleotide (S, lanes c, f, i and l) or 100-fold excess of a non-specific oligonucleotide (N, lanes d, g, j and m) as competitors. Nuclear extracts from each stage were incubated in the presence of anti-GR antibody (BUGR2) (lanes n–q). The arrow indicates the position of the specific GR–DNA complex. (**B**) Mobility shift assays were performed with a ^{32}P-labeled (−73)Col oligonucleotide and nuclear extracts from NF, MF, AF and FS cells (lanes a–d, respectively). The arrow indicates the position of the specific protein–DNA complex, and the asterisk indicates the position of non-specific binding (data not shown). (**C**) Mobility shift assays were performed with a ^{32}P-labeled G(26)A oligonucleotide and nuclear extracts from dexamethasone treated NF (lane b), MF (lane c–e), AF (lane f–h) and FS (lane i–k) cells. Lane a was incubated in the absence of extract. Competition reactions with 50-fold excess of unlabeled G(26)A (S, lanes d, g and j) or 100-fold excess of a non-specific oligonucleotide (N, lanes e, h and k). Both arrows indicate the position of specific protein–DNA complexes, the asterisk indicates the position of non-specific protein–DNA complexes.

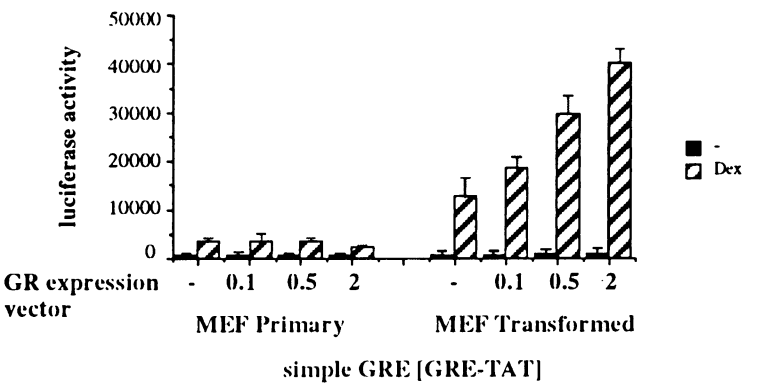

Fig. 8. GR transcriptional activity in primary and transformed mouse embryo fibroblasts. Expression from the TAT3-Luc reporter in primary (left) and transformed (*ras* and SV40 Large T-antigen) (right) mouse embryo fibroblasts (MEF) in the absence or presence of exogenous GR. Cells were co-transfected with the reporter gene and, as indicated, with increasing concentrations (from 0.1 μg to 2 μg) of 6RGR, a GR expression vector. Transfected cells were cultured for 24 h in the absence (solid bars) or presence (hatched bars) of 100 nM dexamethasone. Both cell types displayed comparable levels of β-galactosidase activity.

transformed embryo fibroblasts. As a co-transfected control, levels of β-gal expression from RSV-β-gal were comparable in the two cell types. Thus, our results with embryonic fibroblasts parallel those with dermal fibroblasts and suggest a rather more general distinction between normal and transformed cells.

RAR activity is also low before the fibrosarcoma stage

We have established that the observed differential GR transcriptional regulatory activity is not exclusive to fibrosarcoma development. An additional issue is whether this transition in activity is unique to GR. To address this, we

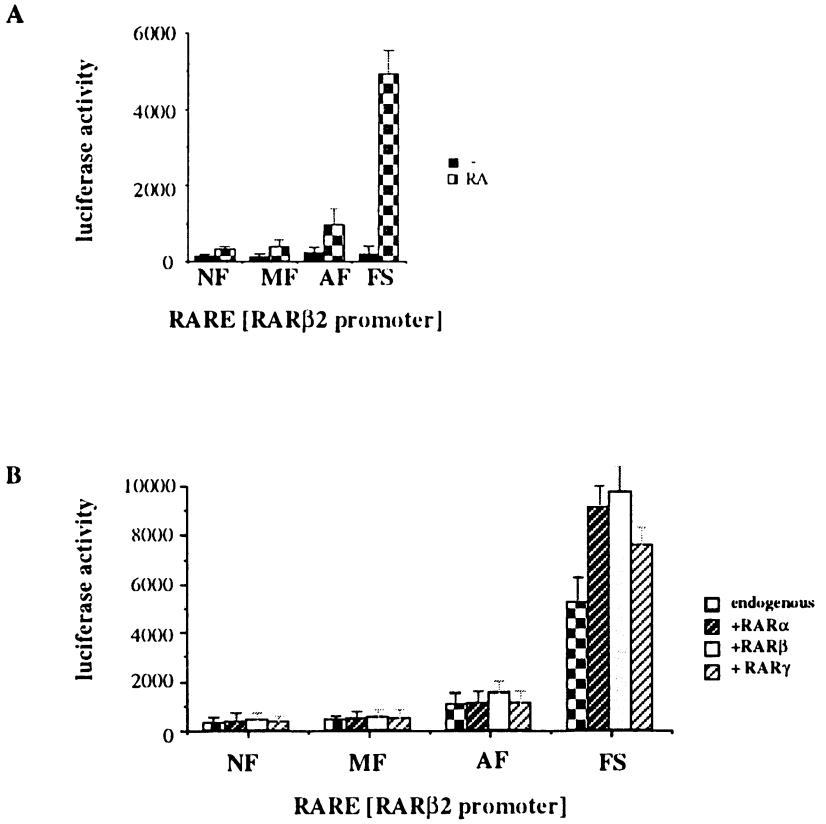

Fig. 9. RAR transcriptional activity during fibrosarcoma progression. (**A**) Expression of the R140-Luc reporter construct in NF 40950, MF 14249, AF BPV3 and FS BPV1 cells. The cells were either untreated (solid bars) or treated for 24 h with 100 nM retinoic acid (checkered bars). Transcriptional activity of endogenous receptor was analyzed. Luciferase activity was normalized to the β-galactosidase activity of a co-transfected reporter. (**B**) Effects of co-transfected RARα, β or γ on hormone responsiveness of cells from the different stages of tumorigenesis. Expression from the R140-Luc reporter in NF, MF, AF and FS cells in the absence or presence of exogenous RARα, β or γ expression vectors. Transfected cells were cultured for 24 h in the presence of 100 nM retinoic acid. A wide range of RAR plasmids (from 5 ng to 5 μg) was tested with similar results; as an example, co-transfection of 0.5 μg of each RAR is shown. β-galactosidase activity was used as endogenous control for efficiency of transfection.

tested an evolutionally distant member of the intracellular receptor superfamily, RAR. We transfected a luciferase reporter construct containing a retinoic acid response element (Vivanco Ruiz *et al.*, 1991), together with the RSV-β-gal expression vector, into NF, MF, AF and FS cells. As shown in Figure 9A, RAR displayed substantially higher activity in the tumor cells than in the pre-neoplastic stages. In contrast, the levels of β-galactosidase expression, used as internal control, were comparable within the different stages (data not shown). As seen with GR (see Figures 5 and 8), co-transfection of expression vectors encoding RARα, β or γ, did not increase the hormonal response in the non-tumor stages (Figure 9B), whereas a modest stimulation was observed in the co-transfected fibrosarcoma cells. These results suggest that the differential transcriptional activity between normal fibroblasts and fibrosarcoma cells is not an idiosyncrasy of GR, and can be extended to other regulators, at least within the intracellular receptor superfamily.

Discussion

A dramatic increase in GR activity at the final stage of dermal fibrosarcoma development

Tumorigenesis is a complex multistep process (for reviews see Hanahan, 1988; Bishop, 1991; Vogelstein and Kinzler, 1993). In the fibrosarcoma pathway considered here, the first pathological stage, mild fibromatosis, expresses the BPV transgenome at low levels. In the next stage, aggressive fibromatosis, BPV transcription increases, aneuploidy develops (Lindgren *et al.*, 1989), angiogenesis ensues (Kandel *et al.*, 1991) and JunB and c-Jun are stimulated, eliciting anchorage independent growth (Bossy-Wetzel *et al.*, 1992). In contrast, other transcription factors such as c-Fos , JunD (Bossy-Wetzel *et al.*, 1992), SP1 and the basic transcription factors acting on the RSV promoter (M.d.M.Vivanco, unpublished data) remain constant during this tumorigenic process. The present study identifies GR and RAR as factors that are specifically up-regulated at the critical fibrosarcoma stage. This differential transcriptional activation is the first molecular parameter that distinguishes the aggressive fibromatosis from the tumor cell, and this transition likely reflects an important step in the mechanism of fibrosarcoma development. In this study we focused a more detailed analysis of this transition on the GR. For example, when pre-neoplastic cells with low GR activity are inoculated into test animals, tumors that arise from those cells display high levels of GR function, consistent with the correlation and with the idea that a switch in GR activity occurs during progression *in vivo*. In contrast, mild fibromatosis cells stably transfected with c-Jun and/or JunB are neither tumorigenic nor do they display strong GR transcriptional regulatory activity (M.d.M.Vivanco, unpublished data).

Importantly, GR is produced and accumulates to the same levels in cells from all four stages of tumorigenesis. Thus, the transition in its activity suggests that some other regulator interacts with GR, either suppressing its activity in the normal and fibromatoses stages, or stimulating its function in the tumor stage. In view of the chromosome rearrangements characteristic of these fibrosarcomas (Lindgren *et al.*, 1989; see Introduction), one could speculate that a suppressor of GR activity might reside on chromosome 14, or that a stimulatory factor gene is on chromosome 8; however, we have not determined whether similar rearrangements are seen in the transformed embryo fibroblasts.

A transition in GR transcriptional regulatory activity

The constant level of GR protein throughout tumor development establishes that the GR transition at the tumor stage reflects increased specific activity, not simply increased receptor production. Furthermore, our immunocytochemistry studies demonstrated that GR from all four stages is localized in the cytoplasm in the absence of hormone and translocates efficiently to the nucleus upon hormone treatment. Consistent with that finding, we showed that a constitutively active GR derivative, N556, which lacks the GR hormone binding domain (Godowski *et al.*, 1987; Miesfeld *et al.*, 1987), is not more active in the pre-neoplastic stages than the hormone-treated full-length GR (M.d.M.Vivanco, unpublished data). Together, these experiments demonstrate that the alteration in GR activity during fibrosarcoma progression must occur after signal transduction events such as hormone entry and metabolism (Funder *et al.*, 1988), GR interaction with Hsp90 (Picard *et al.*, 1990; Bohen and Yamamoto, 1993), hormone binding and nuclear entry.

GR from all four stages in tumorigenesis binds similarly to GRE sequences *in vitro*. This suggests that the modest GR transcriptional activity observed in the normal dermal fibroblasts and fibromatoses reflects a measured capacity of the DNA-bound GR to stimulate the transcription machinery, compared with its robust activity in the fibrosarcoma tumor. This same transition appears to affect the RAR similarly. However, the effect does not reflect a global transition in transcription factor activity, as expression from other promoters, such as RSV or β-actin, does not change during progression, and SP1 functions in all four stages with comparable efficiency. We conclude that the transition in GR activity at the tumor stage reflects a dramatic and selective increase in the receptor potency for transcriptional enhancement, rather than a change in the efficiency of signal transduction.

The target with which GR interacts to effect transcriptional activation is unknown. It is evident, however, that phosphorylation can be an important modulator of the activities of transcriptional regulators (for review, see Hunter and Karin, 1992). GR phosphorylation has been suggested to modulate protein−protein interactions (Orti *et al.*, 1989; Hoeck and Groner, 1990), and it either increases or decreases transcription activation by GR, depending upon the specific sites that are modified (M.D.Krstic, M.J.Garabedian and K.R.Yamamoto, in preparation). In tryptic peptide mapping studies of GR labeled *in vivo* with ^{32}P, we have failed to detect convincing differences in GR phosphorylation during fibrosarcoma progression (M.d.M.Vivanco, unpublished data); further technical advances will be required before we can determine unequivocally whether phosphorylation changes are involved in the GR transition.

Are transcriptional regulators functionally restricted in normal cells?

Viewed from a broad perspective, tumorigenesis can be considered as one form of cell immortalization, and a progression pathway, such as the fibroblast to fibrosarcoma model examined here, as an ordered series in which the process of immortalization can be investigated. Little is known about what distinguishes normal cells, which have restricted capacities for cell division, from established cultured cell lines, or from cells that have undergone oncogenic transformation. In this context, it is notable that mammalian cells are most often studied biochemically in the context of immortalized cells, which can be readily propagated, cloned and manipulated. Indeed, our paradigms for mammalian transcriptional regulatory mechanisms have been established from studies in immortalized cells.

In the case of GR, all previous work suggested that the mere expression of this regulator is sufficient to confer strong transcriptional activation upon promoters bearing linked GREs, and that GR levels limited the magnitude of the activation response. Hence, transfection of mammalian GR into established cell lines from a wide range of tissues and species, including insects (Yoshinaga and Yamamoto, 1991), fungi (Schena and Yamamoto, 1988) and plants (Schena *et al.*, 1991), produced robust regulation upon hormone treatment, and responses that could be amplified further by increasing GR expression. Notably, each study employed immortal cells. Our studies of GR activity in normal and non-immortalized pre-neoplastic cells contrast strongly with those findings: In such cells GR is functional, but confers only modest transcriptional activation (compared with the activity of the same level of receptor at the tumor stage), and overexpression of GR fails to produce increased hormone responsiveness. In addition to the tumor progression pathway, similar conclusions emerged from our comparison of normal and oncogene-transformed mouse embryo fibroblasts (Figure 8), as well as a comparison of normal human keratinocytes with human keratinocytes spontaneously immortalized (containing a mutation in p53) or immortalized with SV40 (M.d.M.Vivanco, unpublished data). In every case, only the immortalized cells displayed strong GR activity and dose-dependent stimulation by exogenous GR. These findings imply that the normal cells and their non-immortalized derivatives may contain a GR modulatory factor, perhaps an enzymatic activity that modifies both endogenous and exogenously provided receptor protein, or a stoichiometric interacting factor present in excess.

According to this view, this same factor would presumably operate on RAR, and its inactivation would release RAR's potent transcriptional regulatory capability. Conceivably, inactivation of such a factor could be associated with the process that regulates cellular immortalization. An important corollary of our postulate is that normal cells may utilize this modulatory factor to restrict the magnitude of transcriptional regulatory signals within

some 'permitted' range. Consistent with this view, many physiological glucocorticoid responses that have been characterized *in vivo* are modest changes in levels of regulated enzymes (Hashimoto *et al.*, 1984; Poduslo, 1989). Clearly, the actions of the putative modulatory factor are selective, as not all regulators are affected, and we would expect that not all cells would be similarly affected. Characterizing the nature of this selectivity will likely be illuminating. In any case, our findings imply that studies of gene regulators carried out using immortalized cells could provide imprecise views of the importance of limiting components, or of the significance of factor interactions and functional cross-talk between factors. This may be an important consideration for investigations that typically have assumed that regulatory phenomena described in established cell lines reflect mechanisms that occur in normal cells.

What is the significance of the transition in GR and RAR activities?

The relationship between the transition in GR and RAR activities and dermal fibrosarcoma development is unknown. Three possibilities merit consideration. First, the transition might reflect a direct role of GR in tumor progression. Glucocorticoid and retinoic acid receptor activities have been correlated with diverse effects on cell growth and differentiation (see Introduction), and it is conceivable that increased GR or RAR function is driving fibrosarcoma progression. This scheme could in principle be tested by treating mice with agonists or antagonists of these hormones, and examining their effects on tumor development and growth. A second model is that the GR and RAR transitions represent a protective cellular response against tumorigenesis that enhances differentiation so as to counteract hyperproliferation. This 'cellular defense' model might explain in part the efficacy of glucocorticoids and retinoids as therapeutic agents in treating certain cancers (for reviews see Smith *et al.*, 1992; Kaspers *et al.*, 1994,). This scheme might also be assessed by pharmacologic studies in whole animals. The third model predicts that GR and RAR are 'bystander' molecules whose transitions to higher activity serve as passive indicators of a key regulatory change in the cell, but that neither receptor is itself directly involved in the mechanism of tumor progression. In all three models, the mechanism by which GR and RAR are up-regulated is a key issue. GR is an especially useful tool for such studies because its functional and physical features are well characterized at the level of biochemistry, genetics, and structure, and sensitive assays are available to measure its activities, or those of a putative GR/RAR-modulatory factor, during tumorigenesis.

In summary, the present study provides new insight into the molecular changes underlying fibrosarcoma formation, with the identification of GR and RAR transcriptional activities as progression markers and potential participants in the oncogenic pathway. In addition, the striking modulation of receptor function in normal and pre-neoplastic dermal fibroblasts may indicate novel controls on intracellular receptors and other regulators not previously observed in studies of transformed cells.

Materials and methods

Plasmids

The luciferase reporter (de Wet *et al.*, 1987) construct TAT3-Luc contained three tandem GREs derived from the tyrosine aminotransferase (TAT) gene (Jantzen *et al.*, 1987) located upstream of the minimal alcohol dehydrogenase (Adh) promoter (−33). The AP-1 reporter construct (−73)Col-Luc, contained a fragment of the collagenase promoter (−73) (Angel *et al.*, 1987) linked to the luciferase reporter gene (B.Kovacic, unpublished results). Three copies of the composite GRE, G(26)A (W.Matsui and K.R.Yamamoto, unpublished results), were placed upstream of the Adh promoter to yield G(26)A3-Luc. The R140-Luc reporter construct contains a fragment (124 to +14) of the RARβ2 gene promoter (Vivanco Ruiz *et al.*, 1991). The GR expression vector 6RGR is a SP65-based vector in which the RSV promoter is fused to GR cDNA sequences (Godowski *et al.*, 1988). The RAR expression vectors contain the corresponding RARα, β or γ cDNAs cloned into a pSG5 vector (Vivanco Ruiz *et al.*, 1991). To monitor transfection efficiency the construct 6RZ (β-galactosidase in a 6R background) (Pearce and Yamamoto, 1993) was included in each transfection.

Cell culture and transient transfections

Cultures were established from skin and tumor tissues of several independent BPV transgenic mice (Sippola-Thiele *et al.*, 1989), and maintained in Dulbecco's modified Eagle's medium (DMEM-21, high glucose formulation; Gibco-BRL) containing 8% fetal calf serum (Hyclone). Cells were propagated for no longer than 5 weeks and always used at low passage number (1–30). Several cultures were tested from each stage of the tumorigenic process: normal fibroblasts, NF (23784, 40950); mild fibromatosis, MF (39614, 14249, 27877); aggressive fibromatosis, AF (BPV7, BPV3, BPV21); fibrosarcoma, FS (BPV1, BPV22, BPV2, BPV11).

Cells were plated at least 12 h before transfection at ~0.5×10^6 cells per 6 cm dish. Reporter gene DNA (2 μg), together with the 6RZ (100 ng) as an internal control for efficiency of transfection, were introduced into cells using the DEAE−dextran method (Ausubel *et al.*, 1993). Where indicated, the expression vector 6RGR was co-transfected. After exposure to the DNA/DEAE−dextran mixture, the cells were incubated for 24 h in fresh medium containing charcoal-stripped serum (Miesfeld *et al.*, 1987) with or without 100 nM dexamethasone (Sigma). Cells were harvested and the luciferase activity measured according to the instructions of the manufacturer (Promega) and normalized for β-galactosidase expression as previously described (Miner and Yamamoto, 1992). Each experiment was repeated at least six times and the results averaged.

Animal and tissue culture of tumor-derived cell lines

Cultured cells to be injected into nude mice were harvested at subconfluency by trypsinization, washed and resuspended in DMEM without serum. Cells in 0.2 ml of DMEM were injected subcutaneously on the right flank of 5–6-week-old nu/nu male mice (Harlan); animals were inspected for tumors at 3 day intervals. Tumors to be re-isolated and cultured were excised following cervical dislocation of the animals. The tumors were washed in phosphate-buffered saline (PBS) and dissociated in collagenase/dispase (Boehringer Mannheim; 10 mg/ml for 30 min, at 37°C). Dissociated cells were then plated and cultured in the same manner as BPV transgenic tumor cell lines.

Immunoblotting

Cells were harvested by centrifugation in 40 mM Tris−HCl (pH 7.8), 10 mM EDTA, 150 mM NaCl, washed in ice-cold PBS, re-pelleted and frozen in liquid nitrogen. Cells were resuspended in lysis buffer [10 mM HEPES (pH 7.9), 400 mM NaCl, 0.1 mM EGTA, 5% (v/v) glycerol, 1 mM dithiothreitol (DTT) and 1 mM phenylmethyl-sulfonyl fluoride (PMSF)]. High-salt extracts were obtained by centrifugation for 30 min at 12 000 r.p.m., discarding the pellet; similar results were obtained using extracts from whole cells solubilized with SDS sample buffer [50 mM Tris−Cl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% (v/v) glycerol].

Equal amounts of extract protein (5 μg) from each cell stage (NF-40950, MF-14249, AF-BPV3, FS-BPV1) were separated by electrophoresis on a 7.5% SDS−polyacrylamide gel and transferred to an Immobilon membrane (Millipore). Blocking, washing and incubation of the membrane with antibodies were carried out in Tris-buffered saline [TBS, 10 mM Tris−Cl (pH 7.5), 150 mM NaCl] containing 4% non-fat dry milk and 0.05% Tween-20. A mouse monoclonal anti-GR antibody

(BUGR2; Gametchu and Harrison, 1984) was used as primary antibody (1:100, hybridoma cell supernatant), followed by incubation with a secondary (horseradish peroxidase conjugated goat anti-mouse immunoglobulin) antibody (1:3000; Bio-Rad). Protein–antibody complexes were visualized by an enhanced chemiluminescence immunoblotting detection system according to the recommendations of the manufacturer (Amersham).

Immunocytochemistry

Cells were grown in 24-well plates (Corning). The medium was changed to serum-free, phenol red-free DMEM, supplemented with insulin (5 mg/ml) and transferrin (10 mg/ml), with or without 500 nM dexamethasone for 2 h. After three washes with ice-cold PBS, cells were fixed with cold (−20°C) methanol for 10 min, and then washed with TBS. Non-specific binding sites were blocked in blocking buffer (BB) (TBS, 1% Triton, 1% glycine, 3% bovine serum albumin and 10% normal goat serum). Fixed cells were treated with a primary rabbit polyclonal anti-GR antibody (PA1–511, 1 mg/ml; Affinity Bioreagents), followed by biotinylated anti-rabbit IgG, and finally horseradish peroxidase, at the dilutions suggested by the manufacturer (ABC, VECTOR laboratories); BB, was used during the antibody incubations, as well as for the washes. Diaminobenzidine (Sigma) was used as a substrate for the peroxidase reaction.

Mobility shift assay

Microscale nuclear extracts from dexamethasone-treated cells (2 h) were prepared as described by Andrews and Faller (1991). Briefly, cell pellet was resuspended in Buffer A [10 mM HEPES–KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF]. The cells were allowed to swell on ice for 10 min and vortexed. Samples are centrifuged and the pellet is resuspended in cold Buffer C [20 mM HEPES–KOH (pH 7.9), 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF] and incubated on ice for 20 min. After centrifugation, the supernatant fraction contained the nuclear extract. Five micrograms of protein from the nuclear extracts were pre-incubated at room temperature for 15 min in 20 mM HEPES–KOH (pH 7.9), 5 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, 15% (v/v) glycerol, 100 ng poly(dI–dC) and 1 mM DTT. After preincubation, 10^4 c.p.m. (3–5 fmol) of the corresponding ^{32}P-end-labeled synthetic oligonucleotide was added to each reaction and incubated for 20 min at room temperature. Protein–DNA complexes were separated by electrophoresis at 200 V at room temperature through a non-denaturing gel [5% polyacrylamide (29% acrylamide, 1% bisacrylamide)] in 0.5× TBE (89 mM Tris–borate, 2 mM EDTA) running buffer. The gel was dried and exposed overnight using Kodak XAR-5 X-ray film.

The following oligonucleotides were used: simple GRE-TAT: 5′-TCGACTGATCTCGCCAGAACATCATGTTCTGCGTCGCCAGGC-3′, AP-1(-73)Col : 5′-TCGACTCTAGACTGAACGGTGACTCAAACTG-CCGCTGCAGGC-3′, composite GRE-G(26)A: 5′TCGACTGATGC-CTGTACAGGATGTTCTAGCTACGAACCCTCGTGAGTCAGTCGA-GGC-3′.

To assess specificity of DNA binding, 50-fold molar excess of unlabeled oligonucleotide [specific (S), same as labeled oligonucleotide], or 200-fold molar excess of a non-specific oligonucleotide (N, a 35 bp oligonucleotide containing completely unrelated sequences from the polylinker of BlueScript; J.Thomas, Ph.D. thesis), were added to the reaction prior to addition of the labeled probe. Where indicated, the anti-GR antibody (BUGR2, 1 μg per reaction; Gametchu and Harrison, 1984) was added to the reaction also before addition of the labeled probe.

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