Microarray analysis identifies Autotaxin, a tumour cell motility and angiogenic factor with lysophospholipase D activity, as a specific target of cell transformation by v-Jun

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We have used chicken cDNA microarrays to investigate gene-expression changes induced during transformation of chick embryo fibroblasts (CEF) by the viral Jun oncoprotein encoded by ASV17. This analysis reveals that v-Jun induces increases and decreases of varying magnitude in the expression of genes involved in diverse cellular functions, most of which have not been detected in previous screens for putative v-Jun targets. In all, 27 individual genes were identified, whose expression is increased threefold or more in v-Jun-transformed cells, including genes involved in energy generation, protein synthesis, and gene transcription. Interestingly, this group includes the hypoxia-inducible factor-1 alpha (Hif-1 α) transcription factor and the glycolytic enzyme enolase, suggesting that adaptation to hypoxia could play a role in tumorigenesis by v-Jun. We also identified 32 genes whose expression is decreased threefold or more, including chaperones, components of the cytoskeleton, and, unexpectedly, DNA replication factors. The gene whose expression is upregulated most dramatically (~100-fold) encodes Autotaxin (ATX), a secreted tumor motility-promoting factor with lysophospholipase D activity. Strikingly, v-Jun-transformed CEF secrete catalytically active ATX and chemotactic activity, which can be detected in conditioned medium. ATX is not detectably expressed in normal CEF or CEF transformed by the v-Src or v-Myc oncoproteins, indicating that induction of this putative autocrine/paracrine factor is a specific consequence of cell transformation by v-Jun. ATX has been implicated in both angiogenesis and invasion, and could therefore play an important role in tumorigenesis by v-Jun *in vivo*.

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Introduction

The viral Jun (v-Jun) oncoprotein encoded by ASV17 induces cell transformation in primary chick embryo fibroblasts (CEF) *in vitro* and fibrosarcomas *in vivo* (Maki *et al.*, 1987). v-Jun is a mutant form of the c-Jun component of the generic transcription factor AP-1 (Nishimura and Vogt, 1988). c-Jun consists of an N-terminal transcriptional activation domain and a C-terminal basic region-leucine zipper (bZIP) DNA-binding domain, and binds to specific TPA-responsive element (TRE) DNA recognition sequences (Angel *et al.*, 1987), either as a homodimer or as a heterodimer with members of the Fos and other families of bZIP partner proteins (see Chinenov and Kerppola (2001) for review). v-Jun differs from c-Jun by three mutations; a deletion of 27 amino acids within the N-terminal transcriptional activation domain and two amino-acid substitutions within or proximal to the DNA-binding domain (Maki *et al.*, 1987; Nishimura and Vogt, 1988). Collectively, these mutations are essential for oncogenesis by v-Jun, since retroviruses encoding c-Jun are nontumorigenic *in vivo* (Bos *et al.*, 1990; Wong *et al.*, 1992).

A large body of evidence supports the view that v-Jun induces cell transformation *in vitro* and oncogenesis *in vivo* via misregulation of cellular target genes; however, the nature and identity of these genes remain ill defined. In part, this is because the relationship between the transcriptional and transforming effects of v-Jun is complex. v-Jun can bind to both TRE and the closely related cyclic AMP-responsive (CRE) elements (Gao *et al.*, 1996); however, the effect on gene transcription is context dependent; v-Jun activates some gene promoters which contain such recognition sequences, but represses others (Gao *et al.*, 1996; Kilbey *et al.*, 1996). Perplexingly, no simple correlation between gene activation and transforming potency has been identified; highly transforming Jun variants can be weak activators and *vice versa* (Havarstein *et al.*, 1992; Morgan *et al.*, 1993). The situation is further complicated by the finding that v-Jun can also affect the expression of genes which do not contain known TRE or CRE sites, presumably by indirect or noncanonical mechanisms (Vogt, 2001). Although these mechanisms

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are not understood, such genes may nevertheless be biologically significant. Finally, only a subset of direct or indirect target genes are likely to play a direct, causal role in cell transformation or tumorigenesis by v-Jun; the remainder may merely be adventitious bystanders (Vogt, 2001).

Despite these uncertainties, a number of genes whose expression is increased or decreased in v-Jun-transformed cells have been identified (see Vogt (2001) for review). Only a minority of these have thus far been proven to be direct targets of v-Jun, while even fewer have been implicated directly in cell transformation or oncogenesis (Vogt, 2001). Heparin-binding epidermal growth factor (HB-EGF) and Jun-activated gene in CEF (JAC), which are known to be direct targets of v-Jun (Fu *et al.*, 1999; Hartl *et al.*, 2001), and TOJ3 (Target of Jun 3; (Bader *et al.*, 2001), are perhaps the best candidates for positive effectors of v-Jun transformation, since ectopic expression of each of these genes alone in normal CEF induces alterations in growth control. With respect to repressed genes, downregulation of SseCKs (Src-suppressed C kinase substrate) may be required for cell transformation, although whether SseCKs is a direct or indirect target of v-Jun has not been established (Cohen *et al.*, 2001).

It also seems likely that v-Jun activates or represses genes which are important for tumor progression *in vivo* but which act on processes, such as angiogenesis or metastasis, which cannot be monitored in cell transformation assays. One potential example of such an occult effector gene is SPARC, which is downregulated in v-Jun-transformed CEF by an indirect mechanism (Vial *et al.*, 2000). Ectopic expression of SPARC has no effect on the growth of v-Jun-transformed CEF *in vitro*; however, the tumorigenicity of the SPARC-expressing cells *in vivo* is greatly diminished (Vial *et al.*, 2000). Taken together, these considerations suggest that many of the effector genes through which v-Jun induces both cell transformation and tumorigenesis remain to be discovered.

cDNA microarrays and oligonucleotide or 'gene chips' provide a powerful means of analysing the global changes in gene expression which underlie complex biological phenomena such as oncogenesis or differentiation. Such methods, however, must be specifically tailored for individual species, and have only recently been developed for use in avian cells (Neiman *et al.*, 2001). As a result, analysis of gene-expression changes induced during v-Jun-induced transformation of CEF, the cell type which most closely represents the natural target for oncogenesis by v-Jun *in vivo*, has relied primarily on subtractive cDNA cloning approaches (Vogt, 2001). While these are effective, they are relatively laborious and require that each differentially expressed gene be identified and analysed individually, thus limiting the number of genes which can be surveyed. In this report, we describe the use of avian glass slide cDNA microarrays (Neiman *et al.*, 2001) to analyse global gene-expression changes during transformation of CEF by v-Jun. Using this approach, we find that v-Jun induces increases and decreases of varying

magnitude in the expression of many genes involved in diverse cellular functions, most of which have not been detected in previous screens for putative v-Jun targets. In addition, it allowed us to identify Autotaxin (ATX), a secreted tumour cell motility and angiogenic factor, which has been shown to potently augment the tumorigenicity of transformed rodent cells *in vivo*, as a specific target of cell transformation by v-Jun.

Results

Microarray analysis of cell transformation by v-Jun

To identify gene-expression changes induced during cell transformation by v-Jun, we compared mRNA samples prepared from control and v-Jun-transformed primary CEF cultures using chicken cDNA glass slide microarrays. The microarrays consist of 3451 cDNA elements representing approximately 2200 individual genes, and have been described previously (Neiman *et al.*, 2001). cDNA probes labelled with Cy3 and Cy5 were prepared using RNA from control and v-Jun-transformed cells, and hybridized in appropriate combinations to separate microarrays to generate dye-reversed replicate data sets. After scanning and normalization (see Materials and methods), the data values from the replicate arrays were merged (averaged) and analysed using Genespring software. A high degree of reproducibility was observed between replicate values for individual cDNA elements. The gene lists and primary data values are available on request.

Figure 1 shows the overall distribution of gene-expression differences which distinguish v-Jun-transformed CEF from their normal counterparts. Approximately 80 individual cDNA elements (~4%) exhibited expression increases or decreases greater than threefold in transformed cells compared to control (above or below the red and blue lines, respectively, in Figure 1), while a much larger number exhibited more modest changes. The most extreme increase observed was around 100-fold and decrease around 10-fold (left panel, arrows). To verify the accuracy of the microarray measurements, a selection of cDNAs showing varying degrees of up- or downregulation (Tables 1 and 2) was used to analyse RNA from control and v-Jun-transformed CEF by Northern blotting. Quantification of this data revealed a good correlation between values established by both methods (Figure 2).

A total of 27 individual known genes (Table 1) were identified among the list of cDNA elements whose expression was increased threefold or more, and 32 whose expression was decreased by a similar factor (Table 2). Inspection of these lists revealed only one, the cathepsin homologue JTAP-1 (Hadman *et al.*, 1996), which was previously known to be deregulated in v-Jun-transformed cells. The remaining cDNAs either displayed little or no significant similarity to any known gene in the public databases or, in a small number of cases, were not associated with reliable sequence information (Neiman *et al.*, 2001). The complete lists

Figure 1 Distribution of genes showing differential expression between v-Jun-transformed and control CEF. Replicate data sets from dye-reversed microarray analyses of mRNA samples from control and v-Jun-transformed CEF were merged and averaged using Genespring software. The distribution of relative expression values for all cDNA elements is shown on the right; genes whose expression is increased in transformed cells compared to control are colored red, decreased blue, while the red and blue lines demarcate genes exhibiting increases or decreases greater than threefold. The range of expression values is shown on the left as a bar graph with the most extreme increase (ATX, Table 1) and decrease (clusterin, Table 2), as indicated by arrows

of these cDNAs and their expression values are available on request.

Upregulated genes

Several of the upregulated genes in Table 1 have been implicated in processes that could contribute to oncogenesis. For example, ATX (107-fold) is a secreted factor that has been shown to stimulate cell motility, invasion, and angiogenesis (Nam *et al.*, 2000, 2001). The relationship between ATX expression and cell transformation by v-Jun and other oncogenes is investigated in more detail below. CD44 (3.1-fold) is a cell surface molecule that has also been implicated in invasion and metastasis, and whose expression has previously been shown to be increased in v-Fos-transformed rat fibroblasts (Lamb *et al.*, 1997). Hif 1 α (6.9-fold) is a transcriptional regulator of oxygen homeostasis, which is thought to play an important role in tumor progression by promoting angiogenesis and hypoxic adaptation (Harris, 2002), while NFkB p50 (5.5-fold) is a transcription factor which can modulate apoptosis and growth control (Chen *et al.*, 2001).

Other upregulated genes are less obviously implicated in oncogenesis; however, some may nevertheless provide useful insights into the biochemistry of cell transformation. For example, increased expression of alpha enolase (3.2–4 fold), a glycolytic enzyme which is regulated in

Table 1 Genes whose expression is increased threefold or more in v-Jun CEF vs control

	Fold increase (no. of elements), clone Id	
<i>Angiogenesis/invasion/cell motility</i>		
Autotaxin	107.3	BI175781
CD44	3.1	DT40subNB_18E5
<i>Energy generation</i>		
Enolase alpha	3.2-4.0 (13)	BE139932
Cytochrome c	9.7	BF845754
<i>Nuclear structure/function</i>		
Nuclear pore protein gp210	8.7	BE139749
Importin 7	3.6-3.9 (3)	BF723884
<i>Protein synthesis</i>		
Elongation factor 1 alpha mRNA	3.4	BE139849
Elongation factor 2	5.0	BE139929
Translation initiation factor 2G	3.3	AW773311
<i>Proteases</i>		
JTAP-1	4.8	pat.pk0040.f3.f
<i>Signal transduction</i>		
MKP-1	4.0	pat.pk0073.f6f
Cytosolic phospholipase a2	4.5	pat.pk0068.f4f
Polo-like kinase 3/ FNK	3.2	pat.pk0049.a12f
IL-16 precursor	9.8	DKFZ426_9N21
IL 8	5.0	pat.pk0023.g8
CD 27L	4.1	pat.pk0049.g3f
CD 33/82	3.1	pat.pk0023.h7f
<i>Transcription</i>		
NF-kB p50 subunit	5.5	DKFZ426_15B16
Hypoxia inducible factor-1 alpha (Hif-1)	6.9	pat.pk0056.f7f
<i>Other genes</i>		
Immunoglobulin J chain	3.3	AW773313
Immunoglobulin light chain	3.3	pat.pk0022.h6
Fatty acid synthase	3.1	pat.pk0060.d3f
VAMP-associated protein B	3.7	pat.pk0067.c7.f0
Gag protein	3.9	pat.pk0067.e4.f0
G0S2	8.2	pat.pk0021.g9
18S ribosomal RNA	3.0, 3.1 (2)	BE140003
28S ribosomal RNA	3.1	BF723916

part by Hif-1 α (Semenza *et al.*, 1996), may be related to the fact that v-Jun-transformed CEF, like most transformed or tumor cells (Dang and Semenza, 1999), exhibit increased glycolysis and excess lactate production (unpublished observations). Likewise, upregulation of translation initiation factor 2G (3.3-fold) and elongation factors 1 and 2 (3.4- and 5-fold, respectively) may indicate that v-Jun-transformed cells have an increased potential rate of protein biosynthesis. This may explain why v-Jun-transformed CEF can divide more rapidly than normal CEF (Clark and Gillespie, 1997), since cell division ultimately depends on mass accumulation and thus on protein biosynthesis.

Downregulated genes

Among the genes whose expression is decreased threefold or more, the proapoptotic Bcl-2 family member

Table 2 Genes whose expression is decreased threefold or more in v-Jun-transformed CEF *vs* control

Fold decrease (no of elements), clone Id				
			v-Jun CEF	Micro-array
<i>Apoptosis</i>				
mtd	0.32	DT40subNB_56		
Clusterin	0.14	pat.pk0059.c2f		
<i>DNA replication/cell cycle</i>				
Ribonucleotide reductase M2 subunit	0.20–0.24 (3)	BE139888		
PCNA	0.18, 0.28 (2)	BE139827		
MCM4	0.25, 0.25 (2)	BF723984		
MCM6	0.25	DKFZ426_19022		
cyclin G1	0.28	pat.pk0073.g12f		
<i>Cytoskeleton</i>				
Actin-related protein Arp3 (ARP3)	0.26	BF723700		
Actin gamma	0.26	pat_12d9		
Myosin light chain (smooth muscle form)	0.29	DKFZ426_21B8		
Clp36 (Lim domain protein)	0.20	pat.pk0053e8f		
Tubulin alpha	0.24	pat.pk0023e5.f0		
Tubulin beta	0.30	BE140004		
<i>Nuclear function</i>				
RAN (TC4)	0.33	BE139947		
Importin alpha	0.29	DKFZ426_7P6		
Nucleolar protein	0.16	DKFZ426_14M1		
Cold inducible RNA-binding protein	0.31	DKFZ426_29J12		
<i>Protein folding/degradation</i>				
HSP60 protein	0.32	DKFZ426_2K3		
Hsc70	0.15–0.33 (7)	BF845717		
Hsc71	0.18, 0.18 (2)	BF845708		
HSP90	0.22–0.33 (3)	BF723928		
Chaperonin CCT delta	0.25	pat.pk0006.f8		
Immunophilin	0.20	pat0039.f4f		
<i>Other genes</i>				
Amyloid precursor	0.29	DT40subNB_7E7		
Ribosomal protein S7	0.24	BF845732		
Cytochrome b5	0.29	BF723821		
Thioredoxin peroxidase II	0.22	pat.pk0071.f5		
Glutathione-dependent PGD synthase	0.24	pat.pk0008.b4		
Annexin VI	0.25	pat.pk0048.c4		
Annexin XI	0.28	pat.pk0056.c6f		
Cathepsin B/Z	0.17	pat.pk0008.b4		
Protein tyrosine phosphatase delta	0.31	ptr1c.2e19		

Mtd (0.32-fold), clusterin (0.14-fold), and the p53-regulated cyclin G1 (0.28) have been implicated in apoptosis (Inohara *et al.*, 1998), tissue remodelling (Jones and Jomary, 2002), and stress responses (Bates *et al.*, 1996), processes which are potentially relevant to oncogenesis. Many of the remaining downregulated genes can be assigned to one of three general functional categories: cytoskeletal components, chaperones, and DNA replication factors. Decreased expression of cytoskeletal components such as actin gamma (0.26), myosin light chain (0.29), and alpha and beta tubulin (0.24 and 0.3, respectively), could contribute to the unusual morphological alterations induced by v-Jun

Figure 2 Validation of microarray expression values by Northern blotting analysis. A selection of cDNA elements showing varying degrees of up- or downregulation by microarray were used as probes in Northern blot analysis of equal amounts (40 μ g) of total RNA from control and v-Jun-transformed CEF. The resulting autoradiograms were quantitated by laser densitometry and the fold increase or decrease in transformed compared to control cells determined. Microarray values given for comparison are derived from Tables 1 and 2

(Cavalieri *et al.*, 1985), while downregulation of the chaperones Hsc70 (0.15–0.33), Hsc71 (0.22–0.33), and HSP90 (0.22–0.33) suggests that cell transformation may be associated with alterations in protein folding or degradation. Decreases were also observed in the expression of ribonucleotide reductase (0.2–0.24-fold), MCM4 and 6 (0.25), and PCNA (0.18–0.28). This was unexpected, since these gene products are required for DNA replication, and previous studies have shown that v-Jun accelerates G1/ S progression and cell proliferation (Clark and Gillespie, 1997; Clark *et al.*, 2000).

v-Jun-transformed CEF secrete catalytically active ATX and chemotactic activity

The gene whose expression was increased most dramatically in v-Jun-transformed CEF was ATX (107-fold by microarray and 47-fold by Northern blotting). These values may be underestimates, since ATX expression was completely undetectable in normal, untransformed CEF by Northern blotting (Figure 2). Owing to this dramatic induction, and because ATX modulates a variety of cellular processes potentially relevant to oncogenesis, we decided to investigate the relationship between v-Jun transformation and ATX expression and activity in more detail.

ATX is an ecto-phosphodiesterase/lysophospholipase D (Clair *et al.*, 1997; Umez-Goto *et al.*, 2002), which is expressed on the cell surface and can be released into the medium via proteolysis (Bollen *et al.*, 2000). Western blotting of cell extracts using an ATX-specific antibody

revealed that v-Jun-transformed CEF expressed readily detectable ATX (ckATX), whereas control CEF did not (Figure 3a). To determine whether v-Jun-transformed cells also released catalytically active ATX, conditioned medium was collected, concentrated, and analysed for ATX protein expression, 5'-phosphodiesterase activity (Clair *et al.*, 1997), and lysophospholipase activity (Koh *et al.*, 2003). As shown in Figure 3b, ATX protein was present in conditioned medium from v-Jun-transformed CEF, but not in medium from control cells. Remarkably, silver staining revealed the presence of a distinct species among the complex mixture of proteins present in the medium from transformed cells (Figure 3c, asterisk) that co-migrated precisely with the ckATX detected by Western blotting, and which was absent from control cell medium. We believe that this

corresponds to ckATX, although this has not yet been confirmed experimentally. Finally, enzyme assays showed that conditioned medium from v-Jun-transformed CEF also contained high levels of 5'-phosphodiesterase and lysophospholipase D activity (Figure 4a).

ATX has been shown to stimulate chemotaxis in a variety of different cell types, and this activity is sensitive to pertussis toxin (Stracke *et al.*, 1992; Lee *et al.*, 2002). We therefore sought to determine whether conditioned medium from v-Jun-transformed CEF contained pertussis toxin-sensitive chemotactic activity in a filter migration assay using NIH3T3 cells as indicators. Recombinant ATX and PDGF were included as examples of pertussis toxin-sensitive and -resistant chemotactic agents for comparison. Conditioned medium from normal CEF was not significantly

Figure 3 v-Jun-transformed CEF express and secrete ATX. Western blot analysis of whole cell extracts (a) and concentrated conditioned medium (b) from control and v-Jun-transformed CEF for expression of chick ATX (ckATX) using an ATX-specific antibody. Purified vaccinia virus-expressed recombinant human ATX (rATX) was included as a control. The apparent molecular masses of rATX and ckATX were estimated to be 105 and 100 kDa, respectively, by comparison to reference markers. Bands in (a) which we believe represent nonspecific crossreacting proteins are indicated (NS). A replicate gel to that shown in (b) was analysed by silver staining (c), revealing that the ckATX protein detected by Western blotting corresponded to one of a series of prominent bands specific to conditioned medium from v-Jun-transformed CEF (asterisk)

Figure 4 Conditioned medium from v-Jun-transformed CEF contains catalytically active ATX and pertussis toxin-sensitive chemotactic activity. (a) portions (10, 20, and 30 μ l) of concentrated conditioned medium from control and v-Jun-transformed CEF were assayed for 5'-nucleotide phosphodiesterase activity and lysophospholipase D activity, as described in Materials and methods. Values (nmol product/reaction) represent the mean and standard error for three independent measurements. (b) Concentrated conditioned medium from v-Jun-transformed CEF was assayed for chemotactic activity in the presence or absence of PTX, as described in Materials and methods. Purified vaccinia virus-expressed recombinant human ATX (rATX), PDGF, and BSA were included as positive and negative controls, respectively. Values (density units, DU) represent the mean and standard error for three independent measurements

chemotactic compared to the negative control, BSA (data not shown); however, the medium conditioned by v-Jun-transformed CEF contained readily detectable chemotactic activity, which, like ATX but unlike PDGF, was substantially inhibited by pertussis toxin (Figure 4b). Taken together, these observations indicate that v-Jun-transformed CEF express and release catalytically and biologically active ATX.

ATX is a specific target of cell transformation by v-jun

To determine whether increased ATX expression is a specific effect of v-Jun, we investigated ATX expression in cultures of CEF transformed by the v-Myc and v-Src oncoproteins. As shown in Figure 5, neither ATX mRNA (a) nor protein (b) was detectably expressed in either, indicating that deregulation of ATX is not a general consequence of cell transformation. We also investigated whether ectopic overexpression of c-Jun induced ATX expression. Cultures of CEF uniformly infected with RCAS cJ-3, RCASvJ-1, and ASV17 (retroviral vectors encoding c-Jun, v-Jun, and gag-v-Jun, respectively; Maki *et al.*, 1987; Morgan *et al.*, 1993) were analysed for ATX mRNA and Jun protein expression. This analysis revealed that ATX expression was induced in CEF expressing both p35 v-Jun and p65 gag-v-Jun, but not in cells expressing exogenous p39 c-Jun (Figure 6a). This suggests that deregulation of ATX depends on one or more of the activating mutations found in v-Jun, although because the level of exogenous c-Jun expression achieved by retroviral expression is lower than v-Jun owing to the greater stability of the mutant proteins (Treier *et al.*, 1994), we cannot rigorously exclude the possibility that a critical threshold level of Jun protein is also required.

Figure 5 ATX is a specific target of cell transformation by v-Jun. (a) Northern blotting analysis of ckATX and GAPDH mRNA expression in total RNA from cultures of CEF transformed by v-Jun, v-Myc, and v-Src. (b) Western blotting analysis of ckATX protein expression in whole cell extracts prepared from the cultures shown in (a)

Finally, we examined ATX regulation in CEF expressing Δ v-JunER, a chimeric v-Jun-estrogen receptor fusion protein which elicits estradiol-dependent cell transformation and transcriptional activation (Kruse *et al.*, 1997). As shown in Figure 6b, ATX was expressed in Δ v-JunER CEF both in the presence and absence of hormone, although somewhat higher levels were evident in estradiol-treated cultures. ATX induction under both conditions was clearly attributable to the action of Δ v-JunER, since no ATX expression was observed in CEF expressing the estrogen receptor hormone-binding domain alone (hER CEF). Similar results were obtained when cells were cultured in phenol red-free medium and charcoal-stripped serum (data not shown), suggesting that induction of ATX in the absence of exogenous hormone was not due to activation of Δ v-JunER by serum-derived estradiol or estrogen mimics. Since the

Figure 6 Ectopic expression of v-Jun but not c-Jun induces ATX expression. (a) Western blot analysis of Jun protein expression (upper panel) and Northern blot analysis of ATX mRNA expression (lower panel) in cultures of CEF infected with the indicated retroviral vectors encoding p39 c-Jun (R c-Jun), p35 v-Jun (R v-Jun), or p65 gag-v-Jun (ASV17). Equal RNA loading was verified by probing for GAPDH (data not shown). (b) Northern blot analysis of total RNA prepared from cultures of CEF expressing the Δ v-JunER, or the estrogen receptor hormone-binding domain alone (hER), cultured in the presence or absence of estradiol

chimeric Δ v-JunER protein contains only the C-terminal DNA-binding domain of v-Jun (Kruse *et al.*, 1997), this implies that one or both of the amino-acid substitution mutations within this region may be critical for induction of ATX gene expression.

Ectopic expression of ATX is not sufficient to elicit cell transformation

The transforming activity of Δ v-JunER is strictly dependent on exposure to estradiol (Kruse *et al.*, 1997; Dunn *et al.*, 2003), indicating that ATX is expressed in these cells regardless of their transformation state. To explore the possible relationship between ATX expression and cell transformation in more detail, we used RCAS, a replication-competent retroviral vector (Hughes *et al.*, 1987), to express human ATX in normal CEF. Two independent CEF cultures infected with RCAS-ATX (Figure 7, 1 and 2) were generated and analysed for ATX expression and evidence of accelerated cell proliferation, a cardinal characteristic of cell transformation by v-Jun (Clark and Gillespie, 1997; Clark *et al.*, 2000). As shown in Figure 7a, Western blotting analysis revealed that both cultures expressed and secreted soluble ATX into the growth medium at levels which were comparable to those released by v-Jun-transformed CEF (Figure 3 and data not shown). The proliferation rate of ATX-expressing CEF, how-

ever, was indistinguishable from vector-infected controls and much slower than v-Jun-transformed CEF (Figure 7b). Furthermore, ATX-expressing CEF did not exhibit any obvious morphological changes or other growth alterations associated with cell transformation by v-Jun such as anchorage-independent growth or continued cell cycle progression in low serum-containing medium (Clark *et al.*, 2000). Although these findings obviously do not rule out a role for ATX in growth deregulation in the context of other gene expression changes induced by v-Jun, ectopic expression of ATX alone is insufficient to induce cell transformation in CEF.

Discussion

Microarrays provide a powerful means of documenting global changes in gene expression; however, it is only recently that it has been possible to apply this approach in avian systems (Neiman *et al.*, 2001). As a result, previous knowledge of gene-expression changes associated with transformation of primary avian cells by the v-Jun oncoprotein has been derived primarily from the use of subtractive cDNA-cloning techniques (Vogt, 2001). Although many interesting and potentially important v-Jun target genes have been unearthed by this route, it is generally agreed that many more remain to be discovered (Vogt, 2001). This is particularly likely to be true for genes which influence complex processes, such as angiogenesis or metastasis, which could play an important role in tumorigenesis by v-Jun *in vivo*, but whose effects cannot be monitored readily in conventional cell transformation assays *in vitro*.

Using chicken cDNA microarrays, we have identified a large number of gene expression differences, which distinguish v-Jun-transformed CEF from their normal counterparts. Interestingly, the overall pattern approximated a normal distribution with around 4% of the genes tested showing increases or decreases greater than threefold, indicating that there is no consistent bias in favor of gene activation or gene repression. In comparison, the number of genes exhibiting more extreme increases or decreases of 10-fold or more was much smaller. Although we cannot determine from this data alone which of these genes are affected directly by v-Jun, for the first time, this analysis gives a clear picture of the overall scale and range of the gene expression differences which distinguish v-Jun-transformed CEF from normal.

With one exception, JTAP-1 (Hadman *et al.*, 1996), the differentially expressed genes which we have identified here, have not previously been associated with cell transformation by v-Jun. The identities of the genes showing changes of threefold or more are remarkably diverse; however, consistent features can be discerned, some of which may be reconciled with the known or inferred characteristics of transformed cells. For example, increased expression of alpha enolase and translation initiation factors, both of which have also been observed in c-Myc-transformed avian cells (Neiman

Figure 7 Ectopic expression of ATX is insufficient for cell transformation. (a) Western blot analysis of ATX expression in concentrated conditioned medium from cultures of CEF infected with RCAS vectors encoding a human ATX cDNA (1 and 2 represent independently derived cultures) or vector control. (b) Proliferation rates of the cultures shown in (a), compared to CEF transformed by v-Jun (RCAS-v-Jun). 2×10^5 cells for each culture were seeded into multiple replicate 90 mm culture dishes, and the number of cells counted at the indicated times

et al., 2001), suggests that v-Jun-transformed cells may have more rapid rates of glycolysis and protein biosynthesis. Increased glycolysis, together with increased expression of the oxygen homeostasis regulator Hif-1 α (and potentially ATX, as discussed below), may indicate that metabolic adaptation to hypoxia constitutes a previously unrecognized facet of the phenotype induced by v-Jun. Interestingly, several recent reports have documented the functional interactions between c-Jun and Hif-1 α (Alfranca *et al.*, 2002; Salnikow *et al.*, 2002).

In comparison, the significance of other consistent gene-expression differences is less immediately obvious. Prominent decreases in the expression of heat shock proteins and other chaperone-like molecules suggest that protein folding or degradation processes and/or stress responses could be perturbed by v-Jun (Beere, 2001). Decreased expression of the DNA damage-inducible and p53-regulated cyclin G1 is intriguing in view of recent observations of genetic and functional interactions between c-Jun and p53 (Schreiber *et al.*, 1999), and could also be indicative of altered stress responses. In contrast, diminished expression of gene products involved in DNA replication, such as ribonucleotide reductase, PCNA, and MCM proteins, seems counter-intuitive, since v-Jun is known to promote cell cycle progression (Clark *et al.*, 2000). The DNA-replication process itself is, however, generally considered to be subordinate to other critical rate-limiting biochemical activities, such as cyclin E/cdk2, which act in G1 to control the onset of Sphase and which are amplified by v-Jun (Clark *et al.*, 2000). Further studies will be required to evaluate the biological significance of these and other changes, and to understand how they relate to the oncogenic mechanism of v-Jun.

The gene whose expression was increased most dramatically encodes ATX, a secreted tumor cell motility factor which was originally thought to be an ecto-phosphodiesterase (Stracke *et al.*, 1992), but which has recently also been shown to possess lysophospholipase D activity (Umez-Goto *et al.*, 2002). The lysophospholipase D activity of ATX can generate lysophosphatidic acid and sphingosine-1-phosphate from appropriate precursors, and it now seems likely that it is through generation of such signalling lipids that ATX exerts many of its biological effects (Moolenaar, 2002; Umez-Goto *et al.*, 2002). Since the increase in ATX expression was so striking, and because ectopic expression of ATX markedly enhances the tumorigenicity of Ras-transformed rodent fibroblasts *in vivo* (Nam *et al.*, 2000), we investigated the relationship between v-Jun and ATX in more detail.

Although ATX is not detectably expressed in normal, untransformed CEF, we find that transformation of these cells by v-Jun results in copious expression and secretion of catalytically active ATX, as judged by the high levels of both phosphodiesterase and lysophospholipase D activity detected in conditioned medium. We believe that the ATX secreted by v-Jun-transformed CEF is also biologically active, since medium conditioned by the transformed cells contains chemotactic

activity which, like ATX, is sensitive to pertussis toxin (Stracke *et al.*, 1992; Lee *et al.*, 2002). On the basis of these results, and the known capacity of ATX to enhance tumor progression (Nam *et al.*, 2000), we believe that it is highly likely that ATX contributes to oncogenesis by v-Jun.

These observations raise two important questions; firstly, whether ATX is a direct transcriptional target of v-Jun, and, secondly, what specific role it might play in tumorigenesis. With respect to the first, it is clear that ATX induction is a specific consequence of cell transformation by v-Jun, since CEF transformed by the v-Src and v-Myc oncoproteins did not express detectable ATX mRNA or protein. Furthermore, ATX was induced by both v-Jun and Δ v-JunER, but not by ectopic overexpression of c-Jun. We believe this indicates that mutations in the v-Jun DNA-binding domain are required for ATX induction, since the Δ v-JunER chimera contains only this portion of v-Jun (Kruse *et al.*, 1997). These observations are consistent with a direct effect of v-Jun on ATX gene transcription; however, because ATX expression in Δ v-JunER CEF was not dependent on estradiol, we were unable to obtain confirm this using a previously described strategy (Fu *et al.*, 1999) of hormonal activation of Δ v-JunER in the presence of protein synthesis inhibitors (EJB, unpublished results). Further work, including identification and functional analysis of the chicken ATX gene promoter, will be required to determine whether ATX is a direct transcriptional target of v-Jun and, if so, to elucidate the exact mechanism of gene activation.

A second important question concerns the potential mechanisms through which ATX might contribute to oncogenesis by v-Jun. ATX has been shown to enhance the tumorigenicity of cells which are already transformed by an activated Ras oncogene (Nam *et al.*, 2000) and to stimulate the growth of human cancer cell lines when added to growth medium in soluble form (Umez-Goto *et al.*, 2002). Ectopic expression of ATX alone, however, is not sufficient induce cell transformation or alterations in growth control in normal rodent fibroblasts (Nam *et al.*, 2001), or as we have shown here in primary CEF (Figure 7, and data not shown). In addition, because ATX is expressed in Δ v-JunER CEF in the absence of exogenous hormone, it is clear that ATX induction does not correlate perfectly with cell transformation by Δ v-JunER, which is strictly dependent on estradiol (Kruse *et al.*, 1997). For both these reasons, we think it is unlikely that ATX mediates v-Jun's effects on cell proliferation.

The other documented biological effects of ATX include stimulation of cell motility, chemotaxis, and angiogenesis, all of which could be important for tumorigenesis *in vivo*. Ectopic expression of ATX can stimulate cell motility in an autocrine or paracrine fashion (Nam *et al.*, 2000); however, in our hands, v-Jun-transformed CEF are less, rather than more, motile than their normal, untransformed counterparts, as judged by time-lapse microscopy and monolayer-wound closure assays (EJB, unpublished observations). Although not definitive, this argues against a scenario

in which ATX stimulates the motility or invasiveness of the transformed cells themselves via an autocrine mechanism. In comparison, the angiogenic properties of ATX appear to stem from paracrine stimulation of endothelial cell chemotaxis and tubulogenesis (Nam *et al.*, 2001). We therefore speculate that ATX may represent an example of a v-Jun-induced paracrine factor which contributes to tumorigenesis via effects on stromal or endothelial cells, perhaps by facilitating tumor angiogenesis or vascularization. If so, induction of ATX by v-Jun may represent an additional adaptation, which enables incipient tumor cells to escape from the constraints of hypoxia *in vivo*. Future studies will seek to test this hypothesis explicitly.

Materials and methods

Cell culture

CEF and CEF transformed by v-Jun, v-Src, and v-Myc were grown in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% new born bovine serum and 1% heat-inactivated chicken serum. NIH3T3 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). For conditioned medium experiments, the cells were grown in serum-free DMEM for 48 h.

Chicken cDNA microarray

The microarrays used in these studies contained 3451 chicken cDNA elements representing approximately 2200 different genes. For the details of the cDNA libraries used and for the construction of the arrays, see Neiman *et al.* (2001). The most recent version of the microarray as used here incorporates approximately 500 additional cDNA elements, and will be described in detail elsewhere (JD and PN, submitted for publication). In Tables 1 and 2, the Genbank Accession number or unique clone identifier is given for each cDNA element; where a single gene is represented by multiple elements (e.g. Enolase), only one identifier is provided, but a complete list is available on request. The origin of the arrayed cDNAs is described in Neiman *et al.* (2001).

Fluorescent cDNA probe labelling, hybridization, and data processing

Total mRNA was prepared from cells by extraction with RNAzol B (Biogenesis) and Qiagen RNeasy columns (Qiagen). Measures of 30 µg of mRNA and 10 µg of oligo-dT were used to generate cDNA in a 30 µl reaction containing 1X Superscript first-strand buffer (Life Technologies), 0.1 M DTT, 25 mM each dATP, dCTP, dGTP, 15 mM dTTP, 10 mM amino-allyl dUTP (Sigma), and 400 U Superscript II reverse transcriptase (Life Technologies). Reactions were incubated for 2 h at 42°C. The RNA was hydrolysed by the addition of 10 µl 1 N NaOH 10 µl 0.5 M EDTA and incubation at 65°C for 15 min. The reaction was neutralized by the addition of 25 µl Tris-HCl pH 7.4 and put through a Microcon 30 filter (Millipore). Cy3 or Cy5 monofunctional dye (Sigma) was coupled to the cDNA by incubation at room temperature in the dark for 1 h. Reactions were quenched by the addition of 4.5 µl 4 M hydroxylamine and incubated at room temperature in the dark for 15 min. The relevant Cy3 and Cy5 reactions were combined and the unincorporated dyes removed using a Qia-quick PCR purification kit (Qiagen). The samples were

dried down and prepared for hybridization by the addition of 18 µl H₂O, 3.6 µl 20× SSC and 1.8 µl PolyA (10 mg/ml), followed by filtration through a 0.45 µm spin column (Millipore). The hybridization of the arrays, image processing, and initial data analysis was as described by Neiman *et al.* (2001). The data were further analysed using GeneSpring 4.0 data-analysis software (Silicon Genetics).

Northern blotting

Total mRNA was prepared as described above. RNA (40 µg) was resolved on 1% MOPS/formaldehyde gels and blotted onto nitrocellulose. cDNAs from the appropriate clones were labelled with ³²P dCTP using a Rediprime II random priming kit (Amersham Pharmacia). The nitrocellulose membrane was hybridized overnight at 65°C in NaPO₄/SDS buffer containing the appropriate radiolabelled probe. After washing, the blot was exposed to an autoradiographic film.

Preparation of cell extracts, polyacrylamide gels, and Western blotting

Whole cell extracts were prepared from cell cultures and resolved by sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE), as described previously (Kilbey *et al.*, 1996; Clark and Gillespie, 1997). Proteins from concentrated, conditioned medium were resolved using 8–16% Novex minigels (Invitrogen), as described previously (Clair *et al.*, 1997). Western blots were carried out in PBS, BSA 1 mg/ml, and Tween20 1%. The anti-ATX antibody was used at 1:1000 and goat-anti-rabbit HRP secondary antibody used at 1:2500–1:5000. Visualization was by chemoluminescence using ECL reagents (Amersham).

Concentration of conditioned medium, purification of recombinant ATX, phosphodiesterase, and lysophospholipase D activity assays

Conditioned medium was prepared by growing cells in serum-free DMEM for 48 h. The medium was concentrated and dialysed into ConA buffer, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM CaCl₂, 20% ethylene glycol, using helium pressure, an Amicon 8400 concentration device, and YM 30 membranes (Millipore), as described previously (Clair *et al.*, 1997). Vaccinia virus-expressed Autotaxin (rATX) was purified and assayed together with conditioned medium for 5'-nucleotide phosphodiesterase activity (determined spectrophotometrically by release of *p*-nitrophenol from *p*-nitrophenol-TMP) and lysophospholipase D activity (determined spectrophotometrically by release of choline from lysophosphatidylcholine), as described previously (Clair *et al.*, 1997; Koh *et al.*, 2003).

Migration assays

NIH3T3 cells were trypsinized and incubated in DMEM, 10% FBS +/– pertussis toxin (PTX) 0.5 µg/ml for 30 min at room temperature, with gentle rocking. The cells were collected by centrifugation, resuspended at 2.0 × 10⁶ cells/ml in DMEM, 0.1% BSA +/– PTX and incubated at room temperature for 30 min with gentle rocking. A volume of 30 µl of attractant in DMEM, 0.1% BSA was put into the lower well of a 48-well Boyden chemotaxis chamber (Neuroprobe, Cabin John, MD, USA) and 56 µl of the 3T3 cell suspension was put into the upper well. The upper and lower wells of the chamber were separated by a gelatin-coated polycarbonate membrane with 8 µm diameter pores. The chamber was incubated for 4 h at 37°C, 5% CO₂. The membrane was fixed and stained in

DiffQuik (Dade Behring Inc., Newark, DE, USA). Nonmigrated cells were removed by wiping the upper surface of the membrane, which was then mounted onto a glass slide. The amount of cell migration was measured in densitometric units using Personal Densitometer SI and ImageQuant (Molecular Dynamics, Sunnyvale, CA, USA). The background OD of cell-free fields was subtracted.

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