Transcriptional profile of Rous Sarcoma Virus transformed chicken embryo fibroblasts reveals new signaling targets of viral-*src*

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Abstract

Transformation of chicken fibroblasts in vitro by Rous Sarcoma Virus represents a model of cancer in which a single oncogene, viral *src*, uniformly and rapidly transforms primary cells in culture. We experimentally surveyed the transcriptional program affected by Rous Sarcoma Virus (RSV) in primary culture of chicken embryo fibroblasts. As a control, we used cells infected with non-transforming RSV mutant td106, in which the *src* gene was deleted. Using Affymetrix GeneChip[R] Chicken Genome Arrays, we report 811 genes that were modulated more than 2.5 fold in the virus transformed cells. Among these, 409 genes were induced and 402 genes were repressed by viral *src*. From the repertoire of modulated genes, we selected 20 genes that were robustly changed. We then validated and quantified the transcriptional changes of most of the 20 selected genes by real-time PCR. The set of strongly induced genes contains vasoactive intestinal polypeptide, MAP kinase phosphatase 2 and follistatin, among others. The set of strongly repressed genes contains TGF beta 3, TGF beta-induced gene, and deiodinase. The function of several robustly modulated genes sheds new light on the molecular mechanism of oncogenic transformation.

Keywords

avian sarcoma; Schmidt-Ruppin strain of RSV; *src* oncogene; protein-tyrosine kinase; vasoactive intestinal polypeptide; MAP kinase phosphatase 2; follistatin

Introduction

Transformation of primary chicken fibroblasts (CEFs) in vitro by Rous sarcoma virus (RSV) continues to represent an important experimental model of cancer (Martin, 2004). The transforming gene of RSV, the viral *src* oncogene (v-*src*), is an activated and over-expressed protein-tyrosine kinase responsible for a number of molecular events that govern rapid and dramatic phenotypic changes observed in transformed host cells (Jove and Hanafusa, 1987;Blume-Jensen and Hunter, 2001;Darnell, 2002;Frame, 2002). Transformation-specific

changes in cell morphology, proliferation and anchorage independence of growth are accompanied by changes in gene expression. In the past, a number of laboratories reported identification of individual genes that were either induced or repressed by the v-*src* oncogene at the level of transcription (Howard et al., 1978;Hendricks and Weintraub, 1981;Fagan et al., 1981;Sugano et al., 1987;Bedard et al., 1987;Jahner and Hunter, 1991;Dehbi and Bedard, 1992;Herault et al., 1992;Frankfort and Gelman, 1995). With the recent completion of the chicken genome sequence (Wallis et al., 2004;Hillier et al., 2004) and the subsequent availability of Affymetrix GeneChip[R] Chicken Genome Arrays that represent the full complement of the genome, we experimentally surveyed the transcriptional program affected by RSV transformation in the syngeneic system of Schmidt-Ruppin strain of RSV (RSV-SR) and primary culture of CEFs. As a control, we used CEFs infected with non-transforming RSV (td106) derived from the Schmidt-Ruppin strain of RSV in which the *src* gene was deleted (Wang et al., 1984). Thus, the isogenic pair of viruses differed only in the *src* gene, but not in the replicative genes, allowing one to score for transcripts relevant for *src* transformation. The rationale for the global analysis of transcriptional changes caused by v-*src* was to provide better understanding of the mechanism of transformation, taking advantage of a cancer model in which a *single* oncogene uniformly and rapidly transforms *primary* cells in culture (Brugge and Erikson, 1977).

Using GeneChip[R] Chicken Genome Arrays that cover 32,773 transcripts corresponding to 28,418 predicted genes from the Ensembl annotation of the complete genome, we report 409 genes that are induced and 402 genes that are repressed at the level of transcription by v-*src*. From the repertoire of modulated genes, we selected 20 genes (10 induced and 10 repressed) that were robustly changed by v-*src* in the screen of Affymetrix DNA arrays. We then validated and quantified the transcriptional changes of most of the 20 selected genes by real-time PCR using array-independent sets of primer oligos and new preparations of RNA. The function of several robustly induced and repressed genes shed new light on the molecular mechanism of oncogenic transformation.

Results and Discussion

Identification of v-src-modulated genes

Analysis of GeneChip[R] Chicken Genome Arrays probed with RSV-SR wild type and RSV-SR-td106 mutant derived probes revealed that circa 10% of transcripts were changed. From 28,418 chicken genes investigated in the screen, 17,868 were detected at least once in RSV-SR or RSV-SR-td106 mutant screens. Among the detected genes, 1602 were induced and 1386 were repressed in v-*src* transformed cells. These changes were considered significant as they had a p-value of 0.05 or less. When a filter of two moderately stringent criteria was applied to the data, namely that the changes had to be more than 2.5 fold (linear, in either direction) and the p-value had to be less than 0.05, the number of modulated transcripts decreased by more than 3 fold to 811. Among the selected transcripts, 409 were induced and 402 were repressed by v-*src* (see Figure A representing a heat map of hierarchical clustering and Table A listing all 811 modulated sequences, in Supplementary Material). This set of data contained several genes that had been previously documented as v-*src*-modulated genes: urokinase-type plasminogen activator, gene for glucose transporter protein, and nephroblastoma overexpressed gene (Leslie et al., 1990;White et al., 1991;Scholz et al., 1996).

Validating selected transcripts by RT-PCR

We chose 20 robustly modulated genes from the "stringent set" of 811 modulated transcripts for further analysis. Among these 20 genes, 10 were induced and 10 repressed (Table 1). We isolated new RNAs from independent RSV-SR transformed and RSV-SR-td106 infected CEFs and generated the array-independent set of primer oligos to validate and quantify the changes

by another technique, real-time PCR (RT-PCR). Using a housekeeping transcript, GAPDH, as a control for quantification, and documenting the quality of each of the amplified products by melting curve analysis, we confirmed most (17 out of 20) of the transcriptional changes identified in the array screen. The changes scored by the GeneChip[R] Chicken Genome Array screen were in most cases larger than the changes measured by RT-PCR, even when the same RNA was used for the assays. This is often the case because of the intrinsic difficulty in setting the right background for the array analysis, especially with low signals from un-induced transcripts (personal communication from T. Hunter).

Gene ontology analysis

In an effort to determine trends in the regulation of classes of genes in response to v-*src* transformation, lists of Affymetrix Probe Set IDs for 811 genes that are significantly increased or significantly decreased (supplemental Table A) were submitted separately to DAVID 2006 (http://niaid.abcc.ncifcrf.gov/home.jsp). DAVID is a program that facilitates the transition from genome-scale datasets to biological meaning (Dennis et al., 2003). The software identified 41 and 47 terms that are significantly over-represented in a functional annotation chart of the genes with increased or decreased expression, respectively. The most significantly over-represented terms for the genes with increased expression included many related to phosphatase activity, proto-oncogenes, nuclear proteins, and DNA-binding. In contrast, the most significantly over-represented terms for the genes with decreased expression included several related to the cytoskeleton, extracellular matrix, cell adhesion, endoplasmic reticulum, signal peptides and glycoproteins. The Probe Set IDs associated with each of these terms are presented in supplemental Table B. The terms associated with down-regulated genes seem to connote normal cellular growth, attachment, and secretion of regulatory factors, while those associated with up-regulated genes hint at abnormal DNA replication. One stand out feature is the number of phosphatases that are up-regulated in the transformed cells.

Integrating and interpreting the transcriptional fingerprint of v-src in CEFs

Two other approaches were elected to analyze the data globally, focusing on the signaling system. In the first approach, we used the most recent version (2.1) of GenMAPP (Gene MicroArray Pathway Profiler) and MAPPFinder, two software packages from Gladstone Institutes of the University of California at San Francisco (www.genmap.org) to analyze changes in the outcomes of signaling pathways caused by v-*src* modulated genes. We uploaded 2988 transcripts that were changed by v-*src*, with p-values no higher than 0.05, into the GenMAPP program and viewed the data on pathway profiles (MAPPFinder) that represent major biological pathways and functional grouping of genes (Doniger et al., 2003). Several signaling pathways, including TGF Beta, MAP kinase, cell cycle, and the kinin-kallikrein component of the complement activation scored well in the GenMAPP analysis in terms of integrating from several to two dozen modulated genes into the signaling schemes. However, the concerted action of changed genes, in terms of activation or repression of the signaling outcomes, was not apparent from this analysis. Therefore, we decided to focus solely on the most robustly changed genes, gathering published data for each one and systematically integrating all of this information into a logical scheme.

Induced genes

After the v-*src* gene, which we found was overexpressed in the RSV-SR-transformed cells on average 265 fold (compared to cellular *src* (c-*src*) gene in RSV-SR-td106 infected CEFs), the urokinase-type plasminogen activator (PLAU) was the second most upregulated gene (196 fold in the array and 42 fold by RT-PCR). Both the PLAU transcript and the protein were previously shown to be elevated in RSV transformed CEFs (Unkeless et al., 1973;Sudol, 1985,Leslie et al., 1990). This enzyme was also implicated in the metastatic potential of tumor cells (Ossowski

and Reich, 1980) and the regulation of fibronectin pathway matrix assembly (Monaghan and McKewon-Longo, 2006).

Vasoactive intestinal peptide (VIP) also belongs to the class of genes that are robustly upregulated by v-*src* (124 fold in the arrays and 53 fold in the RT-PCR) in our screen. As this gene was not known or even expected to be transcriptionally induced in RSV transformed cells, we considered this finding interesting and decided to validate this change further (see below). VIP is a neuromodulator that regulates vasodilation, smooth muscle relaxation and epithelial cell secretion in the intestine. A report on the activation of c-*src* in cells treated with VIP (Koh, 1992) and a recent study implicating VIP in anti-apoptotic signaling (Sastry et al., 2006) could explain the role of this gene in the enhancement and/or maintenance of oncogenic transformation. The frequent occurrence of c-*src* activation in human colon cancer (Bolen et al., 1987;Cartwright et al., 1990;Pories et al., 1998) may be functionally linked to the normal expression of intestinal VIP, which may act as a stimulator of *src* kinase activity (Koh, 1992).

The LOC423497 transcript encodes a protein similar to the human AHNAK (KIAA2019), a high molecular weight (ca. 700 kDa) phosphoprotein that was originally identified as a product of a gene repressed in neuroblastoma cells (Shtivelman et al., 1992;Komuro et al., 2004). One interesting feature of the AHNAK protein is its differential distribution between nuclear versus plasma membrane and cytoplasmic compartments. The nuclear exclusion of AHNAK correlates with cell-to-cell contacts in epithelial cells. The increased expression and the resulting abnormal distribution of the AHNAK in v-*src* transformed fibroblasts may contribute to the loss of contact inhibition (Haase, 2006).

The induction of transcript LOC423769, which encodes an enzyme similar to human arachidonate 5-lipoxygenase (ALOX5), resonates with an earlier study that documented changes in arachidonic acid metabolism in RSV transformed cells (Barker et al., 1989). The transcript is significantly upregulated; 13 fold in the array experiments and 7 fold in RT-PCR assay. ALOX5 metabolizes arachidonic acid into bioactive lipids, which are generally implicated in inflammation and specifically in colon cancer risk (Goodman et al., 2004;Poole et al., 2006). By promoting inflammation, the ALOX5 gene may contribute to the process of tumor progression.

LOC421692 is v-*src* induced transcript that is similar to cAMP-specific 3, 5-cyclic phosphodiesterase 7B (PDE7B). PDEs hydrolize the second messengers, cyclic AMP and cyclic GMP, to their corresponding mono-phosphates, affecting a wide spectrum of intercellular events. Studying this particular isoform of PDE in transformed cells and tumors in more detail may uncover a new aspect of signaling by second messengers, which is relevant for oncogenic transformation.

Follistatin (FST) is a small protein that was originally identified by its ability to inhibit FSH (follicle-stimulating hormone) release by binding to activins, the stimulators of FSH secretion. Activins are members of the TGF beta superfamily of ligands. The activins and their antagonist follistatin act as a pleiotropic growth factor system that regulates cell growth and apoptosis (Stove et al., 2004;Krneta et al., 2006). One possible reason for the increase in follistatin levels in RSV transformed cells could be to titrate out activins in order to keep the TGF beta receptor activity low, eliminating its possible growth-inhibiting signals. Decreased levels of TGF beta 3 and the TGF beta-induced gene, TGFBI, both seen in RSV transformed cells, further support this proposal. Moreover, a recent report showed that follistatin is overexpressed in liver tumors and when recombinant follistatin protein was added to normal or pre-neoplastic hepatocytes in primary culture, it stimulated DNA synthesis (Grusch et al., 2006). In the same system,

addition of activin repressed the synthesis of DNA. In short, follistatin may promote growth of RSV transformed CEFs.

Dual specificity phosphatases (DUSPs) can target various substrates and dephosphorylate them at serine/threonine and tyrosine residues. DUSPs are known to inactivate MAP kinases by dephosphorylating them at specific tyrosine and threonine sites. Perhaps DUSP5 acts in concert with another gene product induced by v-*src*: MAP kinase phosphatase 2 (MKP-2). The increase in the expression of MAP kinase phosphatases is in agreement with previous observations (Stofega et al., 1997). In general, cells stably transformed by v-*src* show only a modest elevation in ERK1/2 activity. However, there is a more marked but transient increase in ERK1/2 activity when a temperature sensitive mutant of v-*src* is reactivated. Furthermore, v-*src*-transformed cells are resistant to ERK1/2 activation by TPA or serum. The elevated expression of MKP-2 and DUSP5 could be a part of a negative feedback mechanism which ensures that ERK1/2 activation is only transient (Stofega et al., 1997).

Repressed genes

NOV (nephroblastoma overexpressed) was originally isolated as a gene that was upregulated in Myeloblastosis Associated Virus-induced nephroblastomas in chickens (Joliot et al., 1992;Martinerie et al., 1994). Similar to the PLAU gene, NOV was also previously shown as a transcript that is modulated by v-*src* in CEFs (Scholz et al., 1996). The NOV (also known as CCN3) gene encodes a secreted protein that is associated with the cellular matrix and promotes cell adhesion. In terms of tumor progression, NOV appears to exhibit suppressive effects in contrast to its related protein, known as CCN (Bleau et al., 2005). Based on the current knowledge of the NOV function in various cells (Lafont et al., 2005;Li et al., 2006;Perbal, 2006), we suggest that the down-regulation of NOV promotes anchorage-independent growth and migration of RSV-SR-transformed CEFs.

Transforming growth factor beta-induced protein of 68 kDa molecular weight (TGFBI) is one of the v-*src* repressed genes. The TGFBI encodes a protein that is similar to fasciclin-like adhesion protein (Kawamoto et al., 1998). The repression of this gene product most likely contributes to the dramatic reduction in adhesive properties of RSV transformed cells. The substantial down-regulation (5–14 fold) of transforming growth factor beta (TGFB3) itself may provide an additional mechanism that ensures a low level of the TGFBI gene product.

Similar to TGFBI, the repression of "four and a half LIM domain 1" protein (FHL1) should also promote non-anchored growth. A recent study has identified FHL1 gene as a tumor suppressor that acts downstream of *src* and CAS (focal adhesion adaptor protein Crk-associated substrate) to block anchorage-independent cell growth and migration (Shen et al, 2006). The c-*src* is known to phosphorylate CAS, which in turn inhibits FHL1 expression and promotes, to a certain degree, cell migration and non-anchored growth. Analogous to the signaling of c-*src*, the v-*src* must phosphorylate CAS quite efficiently to *block* FHL1 expression and to fully enhance non-anchored growth of RSV transformed CEFs. Consistent with this reasoning, FHL1 was found as a gene suppressed in various human tumors (Shen et al., 2006).

The type 2 deiodinase (DIO2) is a seleno-enzyme that catalyzes the conversion of thyroid hormone 4 (thyroxine) to thyroid hormone 3 (triiodothyronine) via deiodination. The repression of this gene in RSV transformed CEFs could lead to the decrease of endogenous triiodothyronine, thereby inhibiting signals of its receptor, cellular erbA. This, in turn, should abrogate inactivation of AP-1 transcription factor as shown previously in chicken cells (Desbois et al., 1991a,b). The active AP-1, together with the upregulated jun (see below), could contribute in unison to signals that enhance cell growth, inflammation and metastasis (Ozanne et al., 2007).

Complement 1q and tumor necrosis factor-related protein 3 (C1QTNF3) is not well characterized in terms of function, and its role in transformation cannot be easily commented on.

In contrast to C1QTNF3, the down-regulation of tissue factor pathway inhibitor gene (TFPI), which encodes a multivalent Kunitz-type serine proteinase inhibitor, could be implicated in the mechanism of transformation. TFPI regulates the extrinsic pathways of blood coagulation. The protein is generally expressed and secreted by vascular cells. Highly malignant human tumors have low or undetectable expression of TFPI compared to benign tumors, implicating the inhibitor in tumor invasion and metastasis (Kondraganti et al., 2006). The activity of TFPI is most likely exerted through plasmin-mediated matrix remodeling. In this light, the acute malignancy of RSV-SR-induced chicken tumors could be explained by the combined action of down-regulated TFPI and up-regulated PLAU.

The model summarizing the concerted action of selected genes that are modulated by v-*src* is presented in Figure 1.

Validating the induction of the VIP transcript at the level of protein

Since up-regulation of the VIP transcript in v-*src* transformed CEF was substantial (ca. 50 fold), we decided to validate its induction at the level of protein. In parallel, we also performed pilot immuno-stainings of human colon and breast cancer biopsies with *src* Tyr-416 phospho-specific and VIP antibodies, hoping to see a correlation between the activated *src* and VIP expression in tumors. Both studies gave us unexpected results. The analysis of VIP protein in RSV-SR transformed cells showed that VIP was also upregulated (9 fold) at the level of protein in transformed cells compared to the control cells that were infected with RSV-SR-td106. However, normal CEFs produced VIP at the level similar to that detected in v-*src* transformed cells (Figure 2). We concluded that RSV-SR-td106 virus repressed the transcription of the VIP gene. The VIP we detected was around 52 kDa, more than twice the size of the prepro-VIP predicted from the coding sequence of the chicken and human gene. The monoclonal antibody recognized the same size, 52 kDa, protein in the chicken and human brain (data not shown). It is possible that the VIP gene product detected in CEFs and in the brain represents a non-processed and non-secreted form of the hormone or may encode a new VIP paralog.

Staining of 12 colon carcinomas and 13 breast carcinoma biopsies identified tumor tissues with clear immunoreactivity for *src* Tyr-416 phospho-specific antibodies (8 in colon adenocarcinoma and 9 in breast carcinoma), yet none of the 25 biopsies showed staining for VIP (Jianhui Shi, Fan Lin and Conrad Schuerch, unpublished data). As expected, the VIP antibody showed staining in ganglion cells residing in normal sections of colon.

General concerns and concluding remarks

The aim of our study was to survey transcriptional changes affected by RSV and its transforming gene, v-*src*, in primary cultures of CEFs. Apart from the basic insight into the process of transformation, we wanted to identify robustly changed transcripts that could serve as cancer biomarkers. Several genes that were not previously known as transcripts changed in transformed cells or sarcomas were identified. Among them, is FST, a hormone-like substance whose protein level could be monitored in human blood.

Two other studies reported analysis of v-*src*-affected genes (Malek et al., 2001; Paz et al., 2004). However, both analyses used a xenogeneic system of chicken v-*src* that was overexpressed in established rodent cell lines, 3Y1 and NIH3T3 lines. Both studies used cells selected with the aid of antibiotic resistance genes for *src* expression, following transfection. In addition, the arrays used in the studies contained a limited number of genes (8,900 human

genes in Malek et al. report; and 12,000 mouse sequences in which 6,000 were non-annotated ESTs in Paz et al. report). Moreover, one of the two studies documented a concordance between transcripts that were present in *src* transformed 3Y1 cells and human tumors, but did not show direct changes between v-*src* transformed and non-transformed 3Y1 cells (Malek et al., 2004). Because of these factors, it is difficult to compare the transcriptional fingerprint of v-*src* transformation of CEFs that we generated to that reported previously in rodent cells, especially in the selected set of 20 robustly modulated genes. With the exception of one robustly down-regulated gene that encodes LIM protein 1 (FHL1), which was also identified by Paz and colleagues (Paz et al., 2004), the remaining 19 genes we reported here (Table 1) were not scored in previous array screens.

We also identified the less robust changes in v-*src* affected transcripts (Table A, Supplementary Material) and consider them important for the understanding of transformation fingerprint. Small changes (two to three fold modulation) in the transcription of genes that encode regulatory proteins or transcription factors *per se* could result in unregulated signaling and contribute to the transformation process (Vogt, 2001). The 3-fold upregulation of jun and myc transcripts was recorded in the array screen. Small changes in the level of transcripts encoding proteins that act in the same pathway could produce synergistic responses. The best example in our data is represented by vascular endothelial growth factor (VEGF) and ribosomal protein S6 kinase. These two genes are up-regulated 3 fold each and their protein products participate in the mTOR pathway (Klos et al., 2006). In RSV-SR transformed CEFs, the level of VEGF gene product is additionally increased by ribosomal protein S6 kinase, which enhances translation of VEGF RNA. The resulting synergy between the two up-regulated gene products may contribute significantly to angiogenesis and metastasis of v-*src* induced tumors. Similarly, we confirmed the upregulation of Eps8 transcript, whose protein serves as a substrate of v-*src* kinase (Gallo et al., 1997). The concerted action of v-*src* and Eps8 should contribute to signals stimulating cell growth.

The transcriptional fingerprint of RSV transformation reported here represents a static and limited view of the transformation process. We have isolated total RNAs from cells that were fully transformed or infected. The cells were harvested soon after they reached complete transformation, as judged by visual inspection. To generate a dynamic picture of transcriptional changes caused by v-*src*, it would be necessary to use temperature-sensitive mutants of RSV. It is likely that certain genes are only modulated at the initial and narrow window of time just when the threshold of transformation by v-*src* is reached (Jakobovits et al., 1984;Jahner and Hunter, 1991). It would also be important to look at genes modulated in RSV transformed cells that were propagated for longer periods of time, allowing adaptation and the epigenetic changes that must occur in spontaneous cancer. Such experiments would allow us to differentiate between genes that are primary targets of v-*src*-induced nuclear signaling pathways and genes that are modulated by the subsequent wave of "secondary" events. Finally, to obtain a more complete picture of transformation, one should connect the repertoire of changed genes with the signaling network of *src* as a *protein-tyrosine kinase* (Blume-Jensen and Hunter, 2001;Feng et al., 2006).

In our survey, we confirmed two out of nine v-*src*-induced genes that were identified in rat fibroblasts transformed by RSV LA23 temperature sensitive mutant (Chen et al., 1977: Jahner and Hunter, 1991). These two genes were rhoB and ornithine decarboxylase. In our assay, both transcripts were elevated only two fold in transformed cells and therefore were excluded from the 'stringent' list of 811 genes. An interesting observation should be noted here. In rat fibroblasts, v-*src* induced a gene, which encoded tissue factor. We did not observe the induction of the tissue factor transcript in CEFs although its probe was present and detected in the array. We did, however, record the repression of tissue factor pathway inhibitor (see Table 1). This single observation could be an example of v-*src* "resourcefulness" in transforming fibroblasts

of different species. *Src* does this by targeting different regulators of the same pathway to achieve the same final "read-out". Host-specific differences in signaling by *src* in chicken versus rodent cells were well documented in the past, and our study further supports this phenomenon (e.g., Hirai and Varmus, 1990).

Interleukin 8 (9E3) and gravin are two genes modulated in RSV transformed cells (Sugano et al., 1987;Bedard et al., 1987;Frankfort and Gelman, 1995). These genes were also scored for in our arrays and their transcripts changed as reported previously, yet the fold changes that we observed were below the two and a half fold cut off.

There are three concerns which need to be addressed in future studies. First, it is important to stress that the mRNA levels do not always correlate with the protein levels (e.g. Tian et al., 2004). Therefore, one needs to show that changes in the level of transcript, which we report here, correspond to changes in the level of protein. Second, the primary culture of chicken embryo fibroblasts is known to also contain small numbers of additional cell types that may respond differently to the RSV infection. The presence of small numbers of myoblasts, neurons, epithelial and perhaps stem cells that 'contaminate' the dominant population of fibroblasts may be responsible for detection of transcripts which seem uncommon for fibroblasts (e.g. nestin). The use of immortalized CEF cell lines such as DF-1 and SC-1 may not provide the best solution to the problem as these cells have unique, immortalization- and transformation-related changes reflected in the transcription of mitochondria-encoded genes (DF-1) or alterations in the p53 and Rb pathways (SC-1) (Kim et al., 2001;Christman et al., 2005). Third, our control virus containing v-*src* deletion (RSV-SR-td106) showed strong activity in repressing the VIP gene in CEFs. Other v-*src*-regulated transcripts could also have been affected by the non-transforming virus; therefore, one should use the primary, non-infected CEFs to control for such a possibility.

In sum, we have identified and confirmed a set of twelve new genes that are highly induced (seven) or repressed (five) in Rous Sarcoma Virus transformed primary chicken embryo cells, compared to cells infected with the control virus with deleted src gene. The quantitative changes and the function of the modulated genes should help us in better understanding the molecular mechanism of oncogenic transformation, which for v-*src* oncogene is characterized by dramatic neoplastic changes of primary cells and high malignancy of resulting tumors. Despite the significant phylogenetic distance of 310 million years between birds and mammals (Burt, 2005), and examples of species-specific differences in signaling between certain orthologs (Van Dyke and Jacks, 2002; Vogelstein and Kinzler, 2006), the human homologues of at least some of the v-*src* modulated chicken genes could represent biomarkers of human cancers (Russello and Shore, 2004;Homsi et al., 2007).

Materials and methods

Cells, Viruses and Preparation of RNA

Chicken embryo fibroblasts (CEFs) were prepared and maintained as previously described (Hanafusa 1969). Three sets of CEFs were infected with high-titer stocks of two viruses. Half of the plates were infected with the transforming, oncogenic virus - Schmidt-Ruppin strain of RSV subgroup A (RSV-SR). The other half were infected with the non-transforming derivative of RSV-SR named td106, in which the *src* gene was deleted (Wang et al., 1984). Both sets of cells were propagated in parallel under standard conditions. When RSV-SR CEFs showed complete morphological transformation (Hanafusa, 1969), the total RNA from the transformed cells and from the control cells infected with td106 RSV-SR was isolated using Qiagen Rneasy Minikit. The RNA was isolated from sub-confluent cultures to ensure that differences in the growth rate between normal and transformed cells were not affected by contact inhibition of normal, confluent CEFs. Yield and quality of RNA were determined through measurement of

the absorbance at 260 nm and 280 nm. All of the RNAs used in the experiments had a 260/280 ratio of 1.9 or higher. These preparations of total RNA served as the template for cDNA synthesis and subsequently for the generation of biotinylated cRNA probes that were used directly in the hybridization to Chicken GeneChip[R] Genome Arrays [Lot # 4009764 and 4017168] (Affymetrix, Inc., Santa Clara, CA).

Microarray Analysis

For each chip, 5µg of RNA was used to synthesize cDNA with an oligo-dT primer attached to a sequence of the T7 promoter region. An in-vitro transcription reaction was used to generate biotin-labeled cRNA from the cDNA. The cRNA was fragmented and an agarose gel was run to verify the sizes of the RNA fragments. A hybridization cocktail was then prepared using 20 µg of the fragmented cRNA. The cocktail was hybridized to a Chicken GeneChip[R] Genome array at 45°C for 18 hr. Next, the cocktail was removed and the arrays were washed and incubated with R-Streptavidin-Phycoerithrin (Molecular Probes, Eugene, OR) following the protocol supplied by the manufacturer. The signal was amplified using a biotinylated antibody raised against streptavidin (Vector Laboratories, Burlingham, CA). The arrays were then washed, stained, and scanned following standard Affymetrix protocols. Each total RNA preparation and its derived cRNA was used to probe one array, giving rise to three experimental values for each chicken transcript that hybridized with the probe. The results were analyzed with the Affymetrix GeneChip Operating Software 2.1 and Spotfire Decision Site 8.1.

Data Analysis

Affymetrix GeneChip Operating Software 2.1 (GCOS) standard adjustments for artifacts, noise and background were used to calculate a detection p-value, detection call and signal for each gene on each array. The entire data set was imported into Microsoft Excel where it was sorted by the detection call. All gene probes scoring a present or marginal on two or more of the arrays in each group were selected for further analysis. This data was imported into Spotfire Decision Site 8.1 where the first analysis was completed. The data set was normalized by scaling each experiment by mean, followed by z-score calculation of the gene probes. The resulting data was then analyzed using a one-way analysis of variance (ANOVA) test to determine which genes were significantly changed. A p-value of ≤ 0.05 was considered significant. The Spotfire Software was then used to cluster the experiments (columns) and gene probes (rows) into a heat map using the unweighted average and Euclidean distance methods. The data was also analyzed using the Affymetrix GCOS software. This consisted of taking the original data from each of the three experiments in the entire data set and comparing them separately. The natural log fold change for each experiment was calculated using the control as the baseline. This data was imported to Microsoft Excel, where the log change was converted to linear change. The final data set consisted of those genes, which had at least a 2.5 linear fold change from the GCOS analysis and a p-value of less than 0.05 from the Spotfire analysis.

Quantitative Real-Time PCR

New total RNAs were prepared from two sets of SR-A-RSV transformed CEFs and two sets of CEFs infected with SR-A-RSV td106. Both sets of RNA from each condition were pooled to make cDNA. The cDNA was synthesized using 2 µg of RNA, 8µl of 5x First Strand Buffer (Invitrogen, Carlsbad, CA), 2µl of 0.1M DTT, 1µl of 50µM Random Hexamers, 0.8µl of 25µM dNTPs, 1µl of Superscript II RT (Invitrogen, Carlsbad, CA), and PCR grade water to bring the final volume of each reaction to 40µl. PCR conditions were 22 °C for 10 minutes, 40 °C for 40 minutes, 99 °C for 5 minutes and 10°C hold. Quantitative Real-Time PCR (RT-PCR) was performed using Quantitect SYBR Green PCR kit (Qiagen, Valencia, CA) as per manufacturer-suggested protocol. A standard curve of serial dilutions (1:1 – 1:100000 of cDNA) and a no template control were included for each primer pair. RT-PCR analysis was

completed using the second derivative maximum method of Roche Molecular Biochemicals Light-cycler Software version 3.5 (Roche, Pleasanton, CA). Using this method, a relative concentration for each sample was generated based on the standard curve of the target of interest. This value was then normalized to the corresponding relative concentration of GAPDH. The resulting normalized data was further used to determine the change in expression of each target by taking the ratio of the experimental value to the control value. Sets of 22-mer-long forward [F] and reverse [R] primers for the chicken GAPDH and the 20 individual transcripts were designed to amplify DNA fragments below 200 bp long. Integrated DNA Technologies Company synthesized and provided the oligo-nucleotides. The primers used in the RT-PCR assay were as follows:

HOUSEKEEPER - GAPDH1-F – AATGCATCGTGCACCACCAACT;

HOUSEKEEPER - GAPDH1-R – TCCCATTCAGCTCAGGGATGACTT;

PLAU-F – TGCAACTTGGACTGGGCAAACA;

PLAU-R – TGCAGGGCGTTTCTTGAATGGA;

VIP_F- TGGGAAACAGACTGCCCTTTGA;

VIP_R- AGCATCAGAGTGGCGTTTGACA;

LOC423497_F-ACATTGCCGTGGAAGCACCAAA;

LOC423497_R-AATCTCACCCGTTGGGAAGTGAGT LOC421581_F-ATCCTGATGTACGCCTGGTTCA;

LOC421581_R-ATTAGGAGAGCAGCAGGCAACT;

LOC423769_F- TTTGGCCAGTATGACTGGTGCT;

LOC423769_R- AACGCTGTTGGGAATGCGGTTT;

LOC421692_F-TGTCTGCATGGCAACAGCATCA;

LOC421692_R-TTGATGAACAGCTGGGTGAGGGAT;

FST_F- TGTGTTTGTGCTCCGGATTGCT;

FST_R- TTTGATCCACCACACACGTGGA;

LOC423890_F- TTGAAGGCTCGTGTTTCGCCTT;

LOC423890_R- TTCAGCAGGATGAAGAAGCCGT;

NES_F- AGCACACCCATTTCCCTTTGGT;

NES_R- AGTGGGAGGGCGATGCTTTATT;

MKP2_F- AAGAAGCGCGTCAAGCTGGAAA;

MKP2_R- AATGGCAGTGTCCGGCACTATT;

NPC2_F- AAGCAGCTGGATAAGAGGGCAT;

NPC2_R-TTGTTGTTCTCCTCCAGTGCCT;

NOV_F-TGCACCAGTGTGCAGACTTACA;

NOV_R-TGAGGACAGCGGAACTCAACTT;

TGFBI_F-GAAGCTTTCCGAGCCATGCCAC;

TGFBI_R -TGGACTTGAGCCGCACAAGAGC;

LOC418180_F-ACGCCGAATGCATCTACACGAA;

LOC418180_R-AGCCAACTTCTCTCATGTGGCA;

LOC428711_F-AAGCAATTGGGTGGGAAGCGTT;

LOC428711_R-TCTTGCAGCCAGCACACTTCTT;

DIO2_F- AAGGGCAACGATGGCAGCAATA;

DIO2_R-TGCACAATGCACACTCGCTCAA;

C1QTNF3_F- ATCAGTTGGCATCTGCTGGCTT;

C1QTNF3_R- TTCTGGAACCTTTGTGGCCAGT;

CPEB1_F-TGCAGCACAAACTAGCCCACAA;

CPEB1_R-ACTGCAGCCAAAGCAGCTTGAT;

TFPI_F-ATGCCATTGCCCTTGGTCTTGA;

TFPI_R-AGTCATCAGTTGCATGCCCAGT;

TGFB3_F-AGAACTGTAATCCAGGAGGCCTGT;

TGFB3_R-TGGGAGCCTTGCAGTTTGCATT.

Immunoblot analysis

Lysates or clarified culture media of uninfected CEFs, of CEFs infected with RSV-SR mutant td-106, and of CEFs transformed by RSV-SR were fractionated on 4–20% pre-cast SDS-polyacrylamide electrophoresis iGels (NG11–420 Life Therapeutics, Australia). Tissues and cells were homogenized in RIPA lysis buffer containing a cocktail of mammalian protease inhibitors. Lysates of adult, normal chicken and human tissues (brain, colon and intestine) were obtained from Zyagen Laboratories (San Diego, CA). Lysate samples: were 25ul/well (CEFs - 40 ug, chicken and human tissues – 100 ug); media samples were: 5ul/well (200 ug). As molecular weight markers we used Kaleidoscope Prestained Standard (161-0324 BioRad, Hercules, CA).

Transfer of resolved proteins to nitrocellulose membrane was monitored by Pounceau red staining. Normalization of proteins was completed on parallel gels and visualized by staining with Coomassie Brilliant Blue R250 solution (Bio-Rad Laboratories, Inc., Hercules, CA) following a standard protocol. After transfer, lysate proteins on nitrocellulose membranes were incubated with anti-VIP monoclonal antibody (SC-25347, Santa Cruz) at 1:200 dilution. The secondary antibody was peroxidase-labeled anti-mouse (NIF825, Amersham) at 1: 5000. After transfer, media proteins on nitrocellulose membranes were incubated with anti-VIP (1:100), with peroxidase-labeled anti-mouse (1:3500). Both blots were then stripped at 65.0°C for 30 minutes, washed 2 times for 10 minutes with TBST containing 0.05% Tween 20 and then blocked one hour at RT in 5% milk. The blots were then incubated with anti-phospho-*Src* antibody (Tyr 416)(2101S, Cell Signaling) at 1:625 dilution followed by the peroxidase-labeled anti-rabbit secondary antibody (1:10,000). ECL cheminoluminescence kit from Amersham (RPN2108) was used for detection of proteins on Western blots.

To quantify the relative differences in the expression of proteins, auto-radiographs of Western blots were scanned using Imaging System unit from Alpha Innotech. Spot Densitometry Quantitation Tool program was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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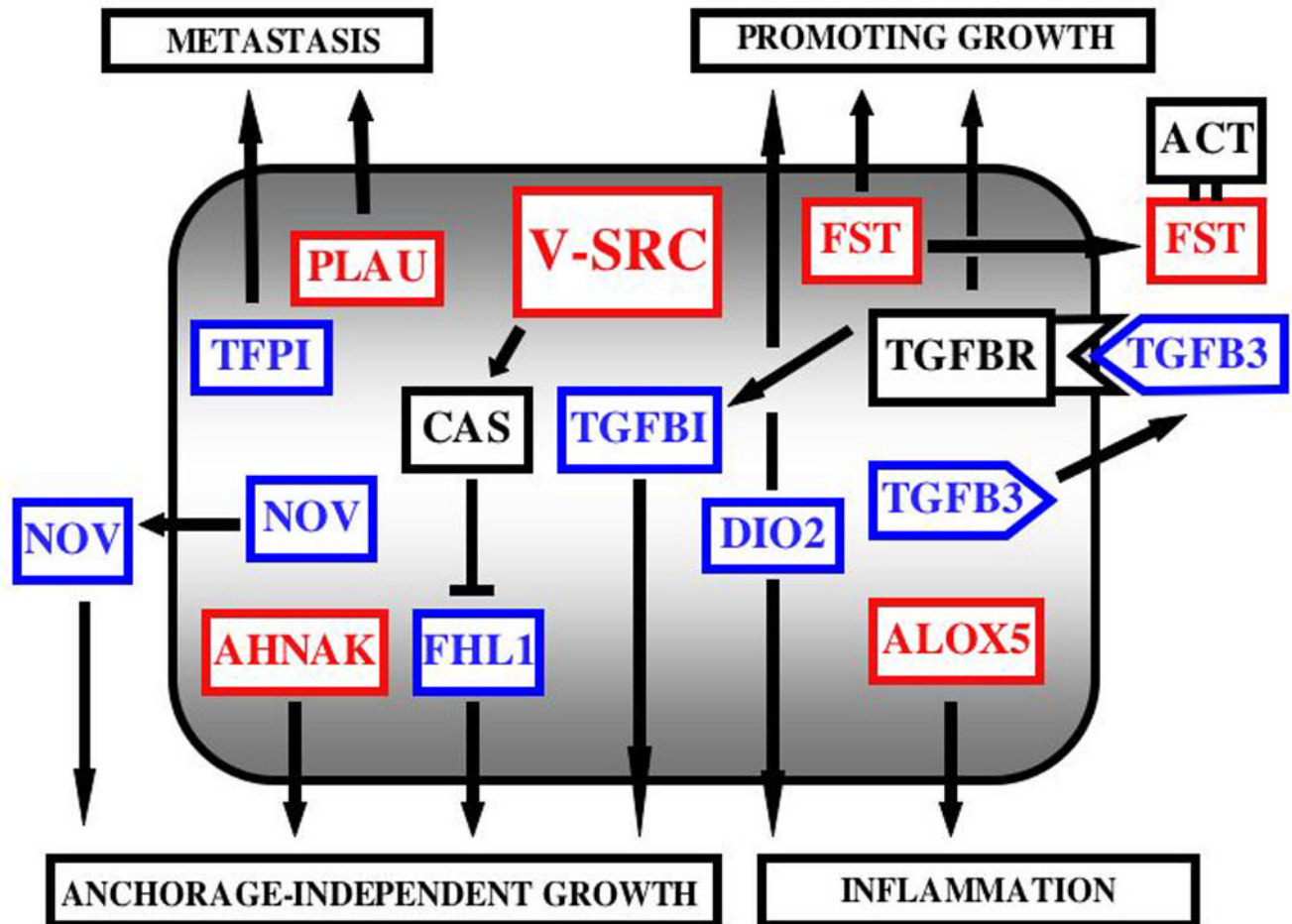
FIGURE 1. Functional network of robustly induced (red) and repressed (blue) transcripts and their interacting partners (black) in primary chicken cells transformed by Rous Sarcoma Virus
By surveying the literature on the function of each of the 20 gene products that were most dramatically changed in RSV transformed CEFs, we propose a model of their concerted action of promoting cellular transformation and tumor progression. Detailed study of individual genes rather than computer-aided annotation of the genes and their networks was employed to reveal functional outputs of changed transcripts, assuming that changes in transcription corresponded to changes in the protein level. Out of the 20 genes modulated by v-*src*, 10 could be linked to promoting a cancer phenotype based on their functional characteristics. These include: Follistatin (FST) that contributes to cell growth stimulation by titrating out activin(s) (ACT) and keeping TGF beta receptor (TGFBR) signaling in "low gear". The low activity of TGFBR is also kept in check by decreased levels of TGF beta 3 (TGFB3). As a consequence, one of the TGF beta-induced genes (TGFBI) that encodes an adhesion protein, is down regulated, contributing to the anchorage-independent growth of transformed cells. Reduced levels of tissue factor pathway inhibitor, (TFPI) together with high levels of urokinase-type plasminogen activator (PLAU), play a role in tumor metastasis. Upregulated arachidonate 5-lipoxygenase (ALOX5) should contribute to tumor development and progression by promoting inflammation. Downregulated type 2 deiodinase (DIO2) would decrease the levels of endogenous tri-iodothyronine and indirectly activate AP-1 transcription factor contributing to signals that enhance cell growth, inflammation and metastasis. The role of four and half LIM domain protein 1 (FHL1) in cell adhesion has been documented and the repression of FHL1,

which is regulated by *src*-phosphorylated CAS, promotes anchorage-independent growth. Tumor suppressive action of nephroblastoma overexpressed gene (NOV) is linked to the control of cell adhesion. The reduced levels of NOV promote anchorage-independent growth. Induction of AHNAK, a gene that encodes a large phosphoprotein, may also contribute to the loss of contact inhibition. See the text for more detailed description of the scheme plus relevant references.
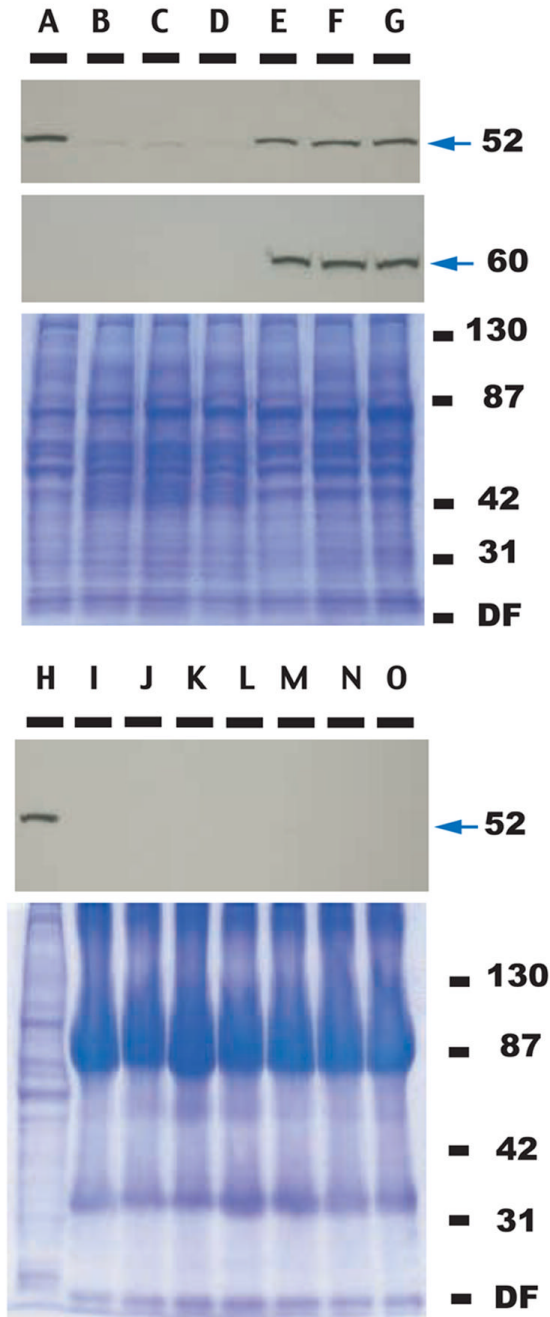
FIGURE 2. Immunoblot analysis of VIP in normal and RSV-SR transformed CEFs
Upper three-partite panel, top panel (lanes A–G): Western blot analysis of cell lysates from CEFs (lane A), CEFs infected with RSV-SR td106 mutant (lanes B, C, D), and CEFs transformed by RSV-SR (lanes E, F, G). The blot was probed with a monoclonal antibody raised against human VIP. Lysates from cells derived from three independent experiments (infection with RSV-SR td106 mutant and transformation with RSV-SR) were used. Arrow points to VIP of 52 kDa molecular weight. Middle panel, the same Western blot was probed with *src* phospho-Tyr 416-specific antibody. Arrow points to c-*src* of 60 kDa molecular weight. Lower panel, Coomassie blue staining of the parallel SDS-polyacrylamide gel analyzed in the upper panels.

Lower two panels, top panel (lanes H–O): Western blot analysis of conditioned media from normal and RSV-SR transformed CEFs; lane H, lysate from normal, uninfected CEFs that serves as a positive control for the presence of VIP; lanes I–K, supernatant from CEFs infected with RSV-SR td 106 mutant; and lanes M–O, supernatant from CEFs transformed with RSV-SR. Arrow indicates VIP migrating at the apparent 52 kDa molecular weight. Lower panel, Coomassie blue staining of the parallel SDS-polyacrylamide gel analyzed in the upper panel.

TABLE 1

TRANSCRIPTS MODULATED IN RSV TRANSFORMED CHICKEN CELLS

GENE SYMBOL	ACCESSION#	MW	GENE NAME	ARRAY(n=3)	RT-PCR(n=2)
			RSV (V-SRC) INDUCED GENES$		
PLAU	NM_205443	49	plasminogen activator, urokinase	196 ± 69	42 ± 11
VIP	NM_205366	22	vasoactive intestinal peptide	124 ± 60	54 ± 0.5
LOC423497	XM_421400	353	*AHNAK 1 & 2 related protein	26 ± 5	8 ± 5
LOC421581	XM_419619	20	*EMT organic cation transporter 3	18 ± 15	N.C.
LOC423769	XM_421641	65	arachidonate 5-lipoxygenase *	13 ± 6	7 ± 3.5
LOC421692	XM_419729	86	cAMP-specific phosphodiesterase 7B *	10 ± 5	10 ± 1.5
FST	NM_205200	38	follistatin	9 ± 5	4 ± 0.5
LOC423890	XM_421754	75	*dual specificity phosphatase 5	7 ± 4	7 ± 1.5
NES	NM_205033	202	Nestin	7 ± 1	1 ± 0.2
MKP-2	NM_204838	41	MAP kinase phosphatase 2	6 ± 2	26 ± 15
			RSV (V-SRC) REPRESSED GENES		
NPC2	NM_001004393	21	stathmin-like 3	45 ± 52	2 ± 0.2
NOV	NM_205268	38	nephroblastoma-overexpressed gene	42 ± 39	18 ± 15
TGFBI	NM_205036	74	TGF beta-induced protein, 68 kDa	28 ± 31	7 ± 4
LOC418180	XM_416411	23	*RAB, estrogen-regulated growth inhibitor	18 ± 8	2 ± 0.3
LOC428711	XM_426269	61	four and a half LIM domains 1 protein	15 ± 1	3 ± 0.5
DIO2	NM_204114	31	deiodinase	15 ± 6	5 ± 1
C1QTNF3	XP_425002	34	C1q and TNF-related protein 3	13 ± 6	3 ± 0.5
CPEB1	XM_413713	80	cytoplasmic polyadenylation element BP1	8 ± 3	0.3 ± 0.1
TFPI	XM_421849	35	tissue factor pathway inhibitor	5 ± 2	3 ± 1
TGFB3	NM_205454	47	TGF beta 3	5 ± 0.8	5 ± 2.5

$
v-src oncogene was upregulated 265 (+/−45) fold in RSV-SR transformed CEF compared to CEF infected with the non-transforming derivative of RSV-SR td106 mutant. Since there is no v-src gene expressed in the control, non-transformed cells, the over-expression value for the v-src transcript reflects a comparison with the endogenous level of c-src transcript. The value is derived from three independent experiments (n=3) in which chicken genome arrays were screened with cRNA probes derived from independent sets of transformed and infected cells.

"MW" represents molecular weight in kiloDaltons of the unprocessed protein product calculated from the translated cDNA of the predicted gene product with the aid of ExPASy tools.

*
denotes "similar" in most cases based on the annotation of the sequence provided by Affymetrix. For all the sequences, which we marked here with '*' and therefore termed as "similar" to known mammalian genes, we performed BLASTP analysis and compared them to human proteins. The sequences were from 60 to 80% identical over the main portions of proteins and most likely represent bona fide orthologs. However, a caution is advised, as there are examples of avian-specific genes that share a significant degree of sequence identity with mammalian genes but do not represent their orthologs, e.g., chicken yrk and human yes (Sudol et al., 1993).

N.C. stands for "not confirmed". It applies to one transcript for which we could not obtain a single size DNA product with the real time PCR, in spite of trying three sets of primers spanning different regions of the sequence.