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Upregulation of transcription factors in lung in the early phase of postpneumonectomy lung growth

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Landesberg, Leonard J., Ramachandran Ramalingam, Kenneth Lee, Todd K. Rosengart, and Ronald G. Crystal. Upregulation of transcription factors in lung in the early phase of postpneumonectomy lung growth. *Am J Physiol Lung Cell Mol Physiol* 281: L1138–L1149, 2001.—In the adult rodent, pneumonectomy results in compensatory lung growth characterized by cell proliferation. The molecular mechanisms governing this response remain unknown. We hypothesized that, in the early period postpneumonectomy, upregulated expression of transcription factors drives the growth process. We utilized a cDNA expression array to screen for upregulated transcription factors after left pneumonectomy in adult C57BL/6 mice, using unoperated mice as controls. Quantification of mRNA expression in the remaining lung at 2 h demonstrated a twofold or greater upregulation of six transcription factors: early growth response gene-1 (Egr-1), Nurr77, tristetraprolin, the primary inhibitor of nuclear factor-κB (IκB-α), gut-enriched Krüppel-like factor (GKLF), and LRG-21. Northern analysis was used to quantify the upregulation of expression of these genes relative to sham thoracotomy and unoperated controls. The largest increase was in Egr-1 (4.7-fold > naive). Time-course analysis over the first 24 h confirmed the transient nature of the early upregulation. In the context that postpneumonectomy lung growth is associated with cell proliferation and that genes such as Egr-1, Nurr77, LRG-21, and tristetraprolin have known roles in stress response, vascular biology, embryology, and cellular development, these data support the concept that transcription factors function early in the cascade of events leading to the compensatory response.

pneumonectomy; postpneumonectomy compensatory response

IN SEVERAL SPECIES of small mammals, the adult lung has the ability to generate a complete growth response after surgical removal of substantial amounts of functional tissue. After pneumonectomy in the rat, mouse, rabbit, and dog, the remaining lung undergoes compensatory hyperplastic growth that results in restoration of the preoperative total lung volume with histologically normal pulmonary parenchyma. This phenomenon, termed postpneumonectomy compensatory lung growth, has been documented to induce expansion of all normal lung parenchymal cell types, including alveolar epithelial cells and capillary endothelial cells, with resulting increases of total DNA, protein, and connective tissue (4, 39). The mechanisms governing the postpneumonectomy response, whether local or humoral, remain unclear, as is the time course of changes occurring at the molecular level. It is known, however, that lung growth in the postpneumonectomy model is associated with both upregulation and downregulation of several classes of genes (7, 8, 13, 23, 34, 36, 52).

Transcription factors, organized into families on the basis of shared motifs, are a diverse group of proteins that regulate cell development, differentiation, and growth by binding to specific DNA sites to modulate gene expression (35). On the basis of the knowledge that multiple transcription factors have defined expression patterns in the developing fetal lung during both airway morphogenesis and alveolarization (5, 15, 20, 56), we hypothesized that the early period after pneumonectomy is likely characterized by an upregulation of expression of transcription factors that act in a signaling cascade that ultimately results in the compensatory growth of the remaining lung. Consistent with this concept, Gilbert and Rannels (13) have demonstrated transient upregulation of the immediate-early genes c-*fos* and *junB* within the remaining lung 30 min after left pneumonectomy, and Dovat et al. (8) have identified the downregulation of expression of putative zinc finger transcription factors in the remaining lung compared with unoperated and sham thoracotomy controls.

Because there are many candidate genes with known functions as transcription factors in both embryonic development and tissue repair, we sought a method for screening the relative expression of large numbers of genes at the mRNA level. We selected an analysis of gene expression using cDNA array technology, allow-

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ing for large-scale comparisons of the expression of multiple genes in a single experiment (33). As an initial approach to this strategy, we utilized a cDNA expression array to screen for upregulated transcription factors in the remaining right lungs of mice within 2 h after left pneumonectomy, a time point before the previously documented period of maximal growth in terms of lung weight, total DNA, and total protein (4, 23, 39). The data demonstrate upregulation of six distinct transcriptional regulatory genes compared with controls.

METHODS

Experimental Animals

Adult male C57BL/6 mice (23–25 g) were divided into three groups: normal control (unoperated), sham operated, and pneumonectomy. Pneumonectomy was performed after the animal was anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The animal was intubated by direct puncture of the trachea using a 24-gauge angiocatheter to function as an endotracheal tube and then ventilated with a Harvard rodent ventilator set at 70 breaths/min and a tidal volume of ~0.3 ml. The animal was then immobilized in the supine position, and the left chest was shaved and cleaned with 95% ethanol. A left thoracotomy incision was made at the fifth intercostal space. The left lung was pulled through the incision and removed after ligation of the left main stem bronchus along with its vascular bundle with a 3.0 silk suture. After ensuring hemostasis, the chest wall, muscle, and skin were closed. A control sham operation included a small left thoracotomy without manipulation of the left lung. The operative time for each animal for all surgical groups was ~10 min. After discontinuation of mechanical ventilation, the animals were observed to resume spontaneous respiration and were then extubated.

Animals were anesthetized at 2 and 6 h and 1, 3, and 7 days after surgery and exsanguinated via transection of the abdominal aorta. The remaining lungs were removed, weighed, and prepared for biochemical and molecular analyses as described below. The lungs from the animals at 1, 3, and 7 days were used to demonstrate the postpneumonectomy lung growth, whereas the lungs at 2 and 6 h and 1 day were used in the assessment of transcription factor gene expression.

Biochemical Analysis

Total genomic DNA was isolated from the right lungs of unoperated control ($n = 5$), *day 7* sham ($n = 5$), and *day 7* pneumonectomy ($n = 6$) mice (50). Immediately after animal death and lung dissection, the entire remaining right lung was completely homogenized in 3.5 ml of lysis buffer (100 mM NaCl, 10 mM Tris·HCl, pH 8.0, and 25 mM EDTA, pH 8.0) by use of a Tissue-Tearor homogenizer (Biospec, Bartlesville, OK) at top speed for 1 min. We then added 10% sodium dodecyl sulfate (SDS) and proteinase K to achieve a final concentration of 2.5% and 0.5 mg/ml, respectively, in a total volume of 5 ml. The lysis mixture was incubated overnight at 37°C. The digestion volume was then adjusted to 10 ml with lysis buffer. After a single extraction of the aqueous phase with phenol-chloroform-isoamyl alcohol (25:24:1; Sigma, St. Louis, MO), total genomic DNA from 400 μl was precipitated with ethanol, washed, dried, and resuspended in 100 μl of 1× TE (40 mM Tris·HCl and 1 mM EDTA, pH 7.4). The concentration of DNA was achieved by spectrophotometry (optical density at 260 nm; OD_{260}), and the total genomic DNA content of the lung was calculated. For all samples, the final ratio of $OD_{260/280}$ was between 1.8 and 1.9.

Total RNA was isolated from the right lungs of unoperated control ($n = 9$), *day 7* sham ($n = 6$), and *day 7* pneumonectomy mice ($n = 6$). After animal death and lung dissection as described above, the entire right lung was completely homogenized, and total RNA was purified with the use of TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. The isolated total RNA was resuspended in nuclease-free water (Ambion, Austin, TX) and quantified by spectrophotometry. The total RNA content of the lung was calculated as described above for total genomic DNA.

Array Analysis

Atlas mouse cDNA expression array membranes (Clontech, Palo Alto, CA) were used for this analysis. Each membrane contains the cDNAs from 588 known genes and 9 putative "housekeeping" genes. The genes are divided into six categories on the basis of known function. Each of the genes has been amplified by polymerase chain reaction (PCR) using gene-specific primers to generate 200–500 base pair products. Each PCR product (100 ng) was spotted in duplicate onto a positively charged membrane. The complete list of the cDNAs as well as their GenBank accession numbers is available on the Internet (http://www.clontech.com/atlas/genelists/index.html).

The Atlas array experiments were carried out with the use of total RNA purified from the right lungs of unoperated control and 2-h postpneumonectomy mice. Total RNA was purified with the use of TRIzol reagent (Life Technologies) as described above and quantified by spectrophotometry. For each experiment, three animals were used from each group, and equal quantities of total RNA were pooled for subsequent analysis.

Thirty micrograms of total RNA from unoperated control and 2-h postpneumonectomy right lungs were treated with amplification-grade DNase I (Life Technologies) in the presence of recombinant ribonuclease inhibitor (Invitrogen, Carlsbad, CA). After extraction with phenol-chloroform-isoamyl alcohol (25:24:1; Sigma), the total RNA was precipitated in ethanol and resuspended in nuclease-free water to a final concentration of 2 μg/μl.

The Atlas array experiment was performed as described in the manufacturer's protocol. Briefly, ^{32}P-labeled cDNAs were synthesized, using 4 μg of DNase-treated pooled total RNA from unoperated control and 2-h postpneumonectomy right lungs by reverse transcription in the presence of [α-^{32}P]-dATP. Oligonucleotide primers representing all 588 target as well as housekeeping control genes on the array were supplied in a premixed solution by the manufacturer. Unincorporated nucleotide was removed by spin column purification (Chroma Spin-200; Clontech). After purification, each of the labeled cDNA mixtures was denatured as described in the manufacturer's protocol and hybridized (2×10^6 dpm/ml) to the Atlas mouse cDNA array membrane at 68°C overnight in ExpressHyb (Clontech) hybridization solution. After overnight hybridization, the membranes were washed three times with 2× SSC (300 mM sodium chloride and 30 mM sodium citrate)-0.5% SDS at 68°C, followed by two final washes with 0.1× SSC-0.5% SDS. The membranes were exposed to X-ray film for 24 h. The pooled total RNA from the three different animals in each group was assessed in duplicate Atlas experiments.

Phosphorimager Analysis

The membranes were mounted and exposed to a phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA) for 5 days. The exposed screen was scanned using the Storm 860 phosphorimager (Molecular Dynamics), and the resulting paired signals representing the expression of each gene on the Atlas blot were quantified using ImageQuant (Molecular Dynamics) software and then tabulated in Microsoft Excel (Microsoft, Redmond, WA). The average background radiographic signal of the unhybridized portions of each membrane was subtracted from the raw expression data to more accurately compare the expression of genes between the two experimental groups. These data were displayed graphically as a scatterplot matrix of the densitometric quantification obtained by the phosphorimager, with the unoperated controls plotted along the abscissa and the 2-h pneumonectomy group plotted along the ordinate (18, 60).

Northern Analysis

The upregulation of expression of several candidate genes coding for transcription factors identified by array analysis was confirmed by Northern analysis. Northern blotting was carried out with the samples from unoperated control, 2-h sham, and 2-h postpneumonectomy right lungs. As described in *Biochemical Analysis*, immediately after animal death by exsanguination, the right lung was dissected free and total RNA was purified using the TRIzol RNA preparation kit from Life Technologies. The isolated total RNA was resuspended in nuclease-free water (Ambion) and quantified by use of spectrophotometry. Three separate experiments were performed using three animals in each group, including the two array experiments as well as an independent set of animals. Total RNA (10 μg/lane) was separated on a 1% denaturing agarose gel, transferred to a nylon membrane (Duralon-UV; Stratagene, La Jolla, CA), and hybridized with [α-^{32}P]dCTP-labeled probes synthesized from cDNA by random hexamer priming (Stratagene). The cDNA probes for the individual transcription factors of interest were generated by PCR amplification of a lung cDNA library by use of oligonucleotide primers obtained from Clontech for use with the Atlas array system. Hybridization was carried out using 10^6 dpm/ml of probe in Quikhyb solution (Stratagene) at 65°C for 1 h. After successive 10-min washes with 2× SSC-0.1% SDS and 0.1× SSC-0.1% SDS, the filters were mounted and exposed to a phosphorimaging screen as described above. The densitometric quantification of the expression of each gene was calculated by phosphorimager as described above. The filters were stripped with 0.1× SSC at 95°C and reprobed with a [α-^{32}P]dCTP-labeled probe against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Ambion) to ensure equal RNA loading and as a control for expression of a putative constitutive gene. The ratio of mRNA expression for each transcription factor of interest relative to GAPDH mRNA was calculated for each animal in the three experimental groups. An equal quantity of total RNA from the lungs of each animal in each of the three experimental groups, unoperated control, 2-h postpneumonectomy, and 2-h sham, was pooled and separated on 1% denaturing agarose gel and transferred to a nylon membrane as described above. Hybridization was carried out with the use of the [α-^{32}P]dCTP-labeled probes described above, and the membranes were exposed to X-ray film.

Time-Course Analysis

To determine the time course of the expression of the genes of interest, pneumonectomy and sham thoracotomy were performed, and animals were killed at 6 h and 1 day (n = 3 for each time point and condition) for Northern analysis as described above. Relative expression of mRNA, as determined by phosphorimager analysis, was quantified and is depicted relative to the unoperated controls.

Statistical Analysis

The data are presented as means ± SE. Comparisons between groups were made by use of the two-tailed Student's t-test.

RESULTS

Postpneumonectomy Compensatory Lung Growth

After left pneumonectomy, the remaining right lungs of adult male C57BL/6 mice demonstrated a compensatory growth increase by weight of ~34% at 7 days relative to unoperated ($P < 0.001$) and sham thoracotomy ($P < 0.001$) control animals (Fig. 1). This growth represented a complete response to the loss of the left lung tissue by pneumonectomy (the left lung represents ~35% of the total weight of both lungs together in unoperated control animals; Ref. 39). Similarly, when analyzed at 7 days after surgery, total right lung DNA was increased relative to unoperated controls by 37% ($P < 0.02$) and to sham controls by 47% ($P < 0.01$). Total right lung RNA was increased by 67% ($P < 0.02$) and 75% ($P < 0.03$) relative to unoperated and sham controls, respectively (Fig. 2). These data support the premise that in the mouse, postpneumonectomy com-

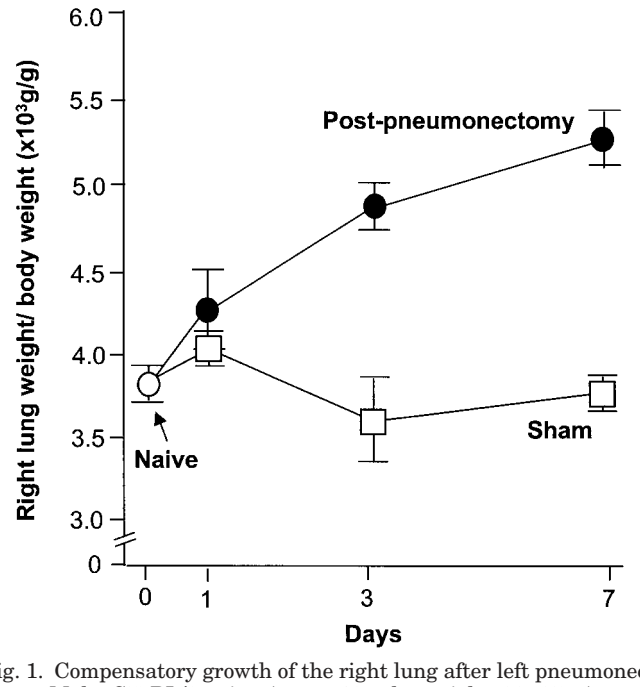

Fig. 1. Compensatory growth of the right lung after left pneumonectomy. Male C57BL/6 mice (age ~10 wk, weight 23–25 g) were subjected to left pneumonectomy or a "sham" procedure consisting of a left thoracotomy without pneumonectomy. The right lung weight-to-body weight ratio was determined at 2 and 6 h and 1, 3, and 7 days. Each data point represents the mean ± SE of at least 6 individual animals/group. The 2- and 6-h data were identical to the naive data (not shown). By 7 days after pneumonectomy, the right lung weight-to-body weight ratio increased significantly compared with sham and unoperated naive controls.

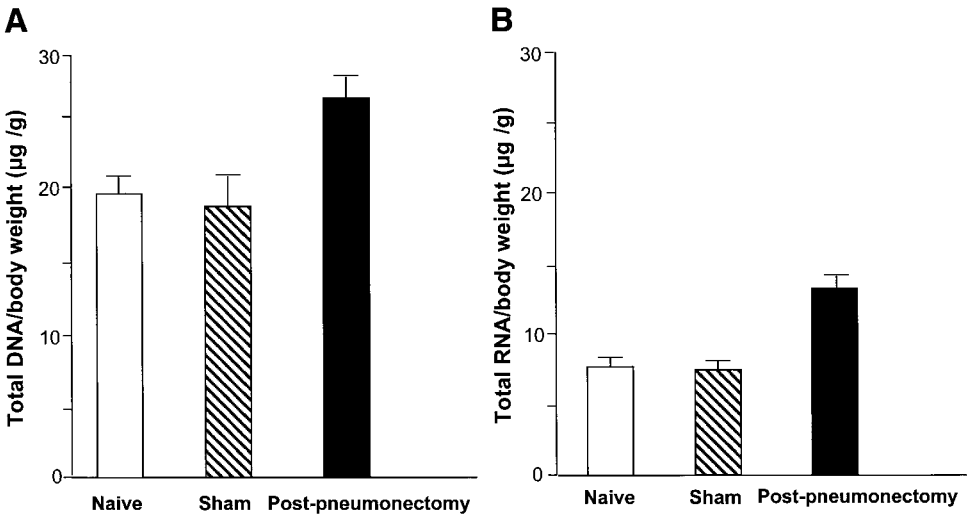

Fig. 2. Total DNA and RNA in the right lung after left pneumonectomy. After death and weight measurements, the excised right lungs were homogenized, and total RNA and DNA were quantified by spectrophotometry. Shown are data 7 days postpneumonectomy. Each bar represents the mean of at least 6 individual lung measurements. *A*: total DNA. *B*: total RNA. Both DNA and RNA increased proportionally to lung weight relative to sham or unoperated naive controls.

pensatory lung growth results in an expansion of total cell number (i.e., a hyperplastic process) rather than by hypertrophy.

Upregulation of Transcription Factors 2 h After Pneumonectomy

The complete Atlas array blot representing the expression patterns of the 588 experimental and 9 housekeeping control genes [ubiquitin, phospholipase A_2, hypoxanthine phosphoribosyl transferase (HPRT), GAPDH, myosin-1, murine ornithine decarboxylase (MOD), β-actin, calcium-binding protein Cab45, and ribosomal protein S29] in the right lungs of unoperated control mice demonstrated expression of a large variety of genes (Fig. 3*A*). The blot was divided into six square sections that contain genes of known similar functions (*top left*: oncogenes, tumor suppressors, cell cycle regulators; *top middle*: stress response, ion channels and transport, intracellular signal transduction modulators and effectors; *top right*: apoptosis, DNA synthesis, repair and recombination; *bottom left*: transcription factors, general DNA-binding proteins; *bottom middle*: receptors, cell surface antigens, cell adhesion; and *bottom right*: cell-to-cell communication, cytoskeleton and motility, protein turnover). The housekeeping and negative control genes were placed in the *bottom* row. Each gene is represented by a set of paired signals on the array. Blots were generated for experiments comparing the mRNA expression patterns of the right lungs of unoperated control mice (Fig. 3*A*) with those 2 h after left pneumonectomy (Fig. 3*B*). To illustrate examples of differentially expressed genes, an enlarged portion of the array (*bottom left*: transcription factors, general DNA-binding proteins) representing both the control (Fig. 3*C*) and 2-h pneumonectomy (Fig. 3*D*) blots demonstrates the paired signals for two upregulated transcription factors [early growth response gene-1 (Egr-1) and gut-enriched Krüppel-like factor (GKLF)].

Scatterplot analysis of the differential expression of the nine putative housekeeping genes by phosphorimaging demonstrated, on average, similar expression 2 h after pneumonectomy compared with naive control (Fig. 4*A*). The data regarding the densitometric phosphorimager measurements for the 588 experimental genes on the Atlas blot comparing 2-h postpneumonectomy lungs with naive lungs were analyzed before average background subtraction (Fig. 4*B*) and after background subtraction (Fig. 4*C*). This subtraction was performed to identify the differential expression of genes, the expression values of which on the blot could only be measured as slightly above background (i.e., genes, the expressions of which varied only slightly above background, might truly demonstrate significant differential expression when compared after subtracting the background from analysis). The slopes of the best-fit lines through the scatterplots were 0.83 before subtraction analysis (Fig. 4*B*) and 0.79 after subtraction analysis (Fig. 4*C*). Thus the global differential expression for the entire blot was ~80%. These data suggest that in the right lungs of mice 2 h after left pneumonectomy compared with unoperated controls, there is a relative downregulation of expression of the majority of genes from baseline. Indeed, comparison of the individual signals on the array blots demonstrates many "downregulated" genes (Fig. 3, *A* and *B*).

To minimize the chance of false positives and to identify only the most significantly upregulated transcription factors 2 h after pneumonectomy, the phosphorimager densitometric values after background subtraction were graphed as a $\log_{10}$ scale plot (Fig. 4*D*). The parallel dashed lines represent twofold up- or downregulation from the best-fit line. Candidate genes for further analysis were selected if they demonstrated greater than or equal to twofold upregulation in two independent Atlas experiments (18, 46, 60). As an example, a candidate gene (Egr-1) is illustrated as lying above the twofold line on the $\log_{10}$ plot.

Northern Analysis

From the Atlas screen, six transcription factors were identified as demonstrating at least twofold upregulation in the postpneumonectomy lung compared with

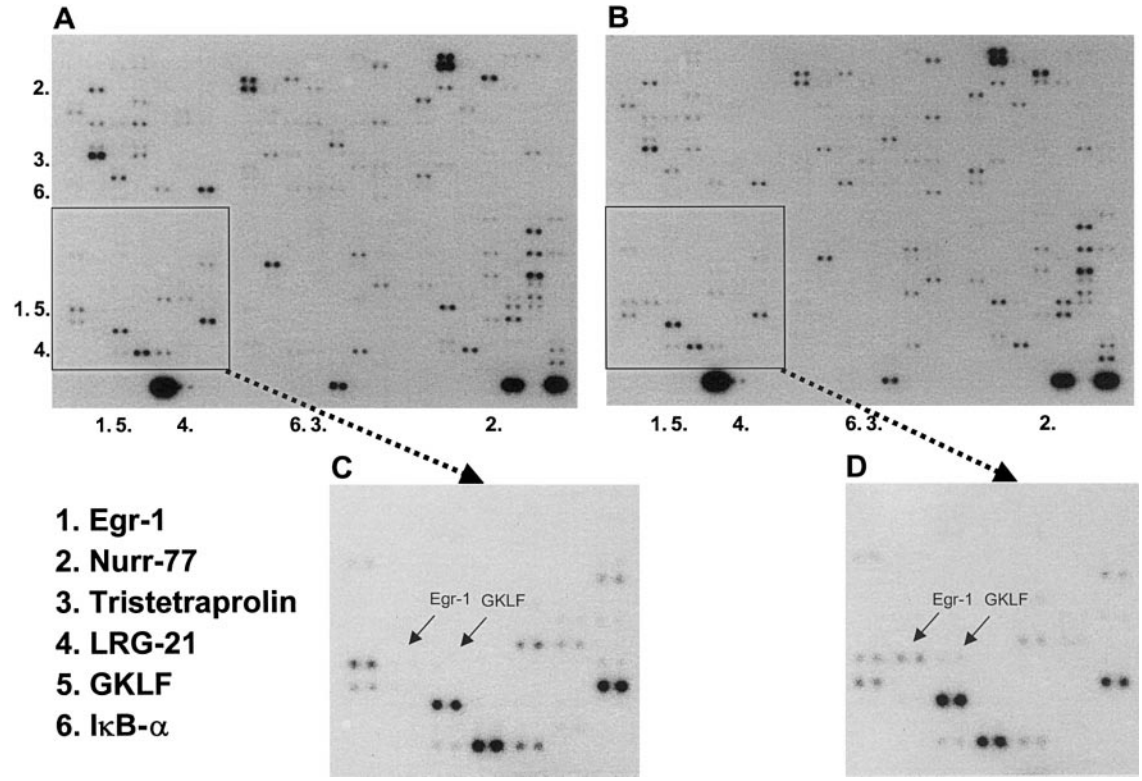

Fig. 3. Atlas array blots demonstrating upregulation of genes in the lungs of 2-h postpneumonectomy mice relative to unoperated controls. An equal quantity of total RNA extracted from the pooled lungs of 3 pneumonectomy and 3 unoperated control mice was reverse transcribed, and the radiolabeled cDNA probes were hybridized to the Atlas membrane. Overnight exposure to autoradiographic film demonstrated multiple signal pairs corresponding to individual genes on the array. *A*: autoradiograph of the entire array for the unoperated control blot. *B*: 2-h postpneumonectomy cDNA array blot. The blots are organized into 6 square regions that roughly denote the known functions of each gene in the group. Each gene is denoted numerically on the respective blots as the intersection of a horizontal and vertical line between identical numbers. The key is located at the *bottom*. *C*: enlargement of a characteristic section of the unoperated control blot. *D*: the same section of the 2-h postpneumonectomy blot. The arrows indicate 2 genes [early growth response gene-1 (Egr-1) and gut-enriched Krüppel-like factor (GKLF)] that are clearly upregulated in the 2-h group relative to the controls. Note that transcription factor LRG-21 is not visible on the overnight autoradiographs. This signal was clearly detectable by phosphorimager analysis in the 2-h blot (not shown). IκB-α, primary inhibitor of nuclear factor-κB.

naive lung in two independent Atlas experiments, including Egr-1, Nurr77, LRG-21, tristetraprolin, GKLF, and the primary inhibitor of nuclear factor (NF)-κB (IκB-α). Northern analysis was then used to confirm the levels of increased expression predicted by phosphorimager analysis of the signals on the blots and to include sham thoracotomy as an additional control (Figs. 5 and 6). GAPDH mRNA expression was used both as a constitutive control gene and to normalize for RNA loading.

After pneumonectomy, Egr-1 demonstrated a 4.7-fold upregulation at the mRNA level relative to unoperated controls ($P < 0.01$) and 3.2-fold upregulation compared with sham thoracotomy ($P < 0.05$). Although the expression of Egr-1 was mildly enhanced in the sham animals relative to the unoperated controls, this was not significant ($P > 0.07$; Fig. 6*A*). Nurr77 mRNA was significantly upregulated (2.9-fold) after pneumonectomy relative to control animals ($P < 0.02$), and the results of sham thoracotomy were not different from the unoperated group ($P > 0.8$; Fig. 6*B*). LRG-21 mRNA was significantly upregulated (1.9-fold) in the pneumonectomy group relative to the sham surgery ($P < 0.01$) and naive groups ($P < 0.01$; Fig. 6*C*). Pneumonectomy induced an upregulation of tristetraprolin mRNA by 3.2-fold ($P < 0.01$) relative to unoperated controls; sham thoracotomy also induced a significant upregulation of 2.2-fold compared with the naive controls ($P < 0.01$; Fig. 6*D*). GKLF mRNA was upregulated 1.9-fold after pneumonectomy ($P < 0.01$) and 1.3-fold after sham thoracotomy ($P > 0.2$) compared with naive controls (Fig. 6*E*). The difference between the pneumonectomy and sham groups only approached statistical significance ($P < 0.06$). Relative to unoperated controls, pneumonectomy also induced a significant upregulation (2-fold) of IκB-α mRNA ($P < 0.01$), but this was not significantly different from the sham animals ($P > 0.6$; Fig. 6*F*).

Time Course

A time-course analysis over 24 h after left pneumonectomy demonstrates that at the mRNA level, the six transcription factors described above rapidly return to

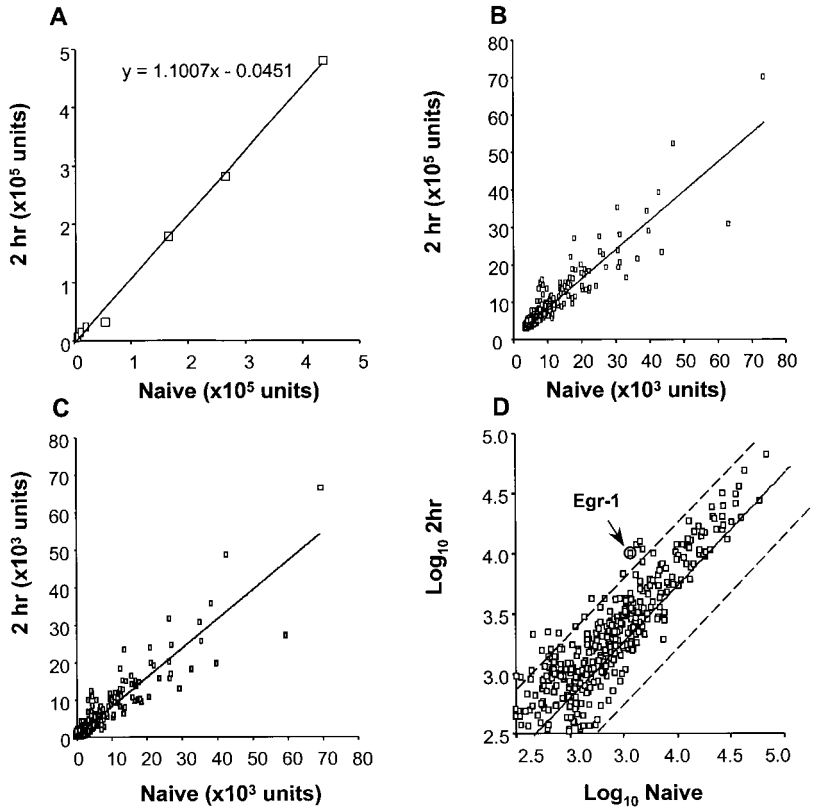

Fig. 4. Phosphorimager analysis of the hybridization signals generated by the Atlas array demonstrating upregulated genes in the lungs of mice 2 h after left pneumonectomy. The array blots assessed as described in Fig. 3 were quantified by use of ImageQuant software. The results were tabulated in Microsoft Excel and analyzed to identify upregulated genes at the mRNA level. The results are displayed graphically as a scatterplot matrix of the densitometric quantification obtained by the phosphorimager, with the unoperated controls plotted along the abscissa and the 2-h postpneumonectomy group plotted along the ordinate. *A*: phosphorimager signals of 9 designated housekeeping genes [ubiquitin, phospholipase A_2, hypoxanthine phosphoribosyl transferase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), myosin-1, murine ornithine decarboxylase, β-actin, calcium-binding protein Cab45, and ribosomal protein S29] are plotted. Those housekeeping genes were used to normalize the expression ratios between different array plots. The slope of the "best-fit" line through the data points is ~1. This allows the relative "expression" values obtained for each gene to be compared without a significant correction for the specific activity of the radiolabeled cDNA probes. *B*: phosphorimager signals of each of 588 genes on the array plotted before subtracting the average background signal (average densitometric quantification of ~100 unoccupied loci on each blot). *C*: phosphorimager signals of each of the 588 genes on the array after subtracting the average background signal of each blot. *D*: $\log_{10}$ scale plot after subtracting the average background. The arrow designates an example of a transcription factor (Egr-1) that falls above the best-fit line and demonstrates an at least 2-fold upregulation (dashed line) over the unoperated controls. The candidate genes showing ≥2-fold upregulation were confirmed visually by inspection of the original autoradiogram or phosphorimage. (Note that the best-fit lines in *C* and *D* demonstrate a slope of ~0.8, suggesting that, at the mRNA level, most of the genes on the array do not change dramatically in the remaining lung 2 h after pneumonectomy compared with an unoperated control.)

baseline (Fig. 7). This effect was evident by 6 h, and although the trend suggests that three of the genes (tristetraprolin, GKLF, and IκB-α) remained upregulated relative to controls, these differences were not statistically significant.

DISCUSSION

This study is based on the hypothesis that the early phase of postpneumonectomy compensatory lung growth is accompanied by an upregulation of a variety of transcription factors. To assess this hypothesis, cDNA array technology was used for screening of candidate genes, and Northern analysis was used to validate the expression of genes for transcription factors that may play a role in this complex process. Relative to unoperated controls, the remaining right lungs of C57BL/6 mice killed 2 h after left pneumonectomy demonstrated a significant upregulation at the mRNA level of six transcription factors, including Egr-1, Nurr77, LRG-21, tristetraprolin, GKLF, and IκB-α, although the upregulation of IκB-α was not different than with sham surgery.

Postpneumonectomy Compensatory Lung Growth in the Mouse

The data demonstrate compensatory growth of the remaining right lungs of adult male C57BL/6 mice as reflected by an increase in right lung wet weight-to-

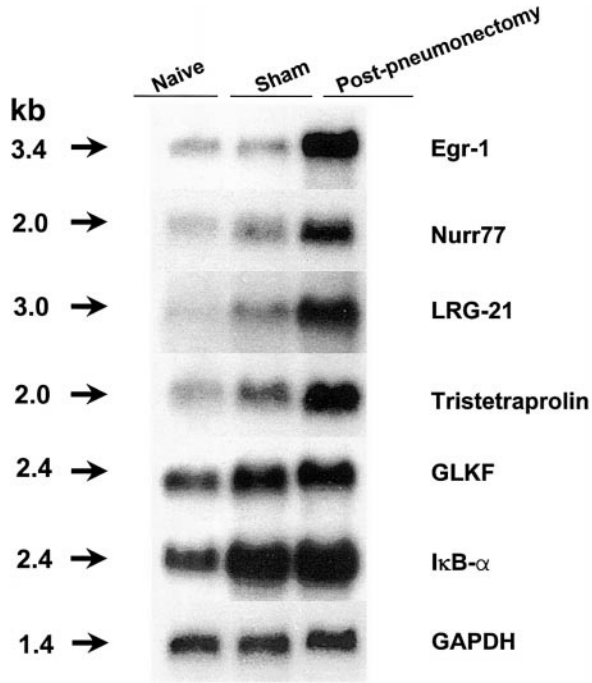

Fig. 5. Confirmation of the upregulation of mRNA expression in the right lung 2 h after pneumonectomy for each identified transcription factor by Northern blotting compared with expression in sham-operated control animals. Pooled total RNA from the right lungs of 3 male C57BL/6 mice (23–25 g) in each group (unoperated naive controls, 2-h sham, and 2-h postpneumonectomy) was assessed by Northern hybridization performed using [^{32}P]dCTP end-labeled cDNA probes for the genes of interest. Equal RNA loading was confirmed by analysis of GAPDH mRNA. The estimated size of the mRNA transcripts is shown at *left*. Expression at the mRNA level was clearly upregulated in the pneumonectomy group relative to the unoperated controls. Some of the genes also demonstrated upregulation in the sham group.

body weight ratio and by an increase in total lung DNA per body weight and total lung RNA per body weight at 7 days postpneumonectomy. These growth results are consistent with the published data in several species, including the mouse and rat, in which the complete response occurs over ~7–14 days (39).

Assessment of Transcription Factors Postpneumonectomy

Although the time course and extent of postpneumonectomy compensatory lung growth have been well characterized at a morphological level, the process is not well understood at a molecular level (39). We hypothesized that upregulation of transcription factors likely plays a role early in postpneumonectomy lung growth. Therefore, we chose to screen for an upregulation of expression of transcription factors 2 h after pneumonectomy in the mouse model. The time point of 2 h was selected on the basis of the observation that by 90 min, the mice had emerged from anesthesia and by 2 h, their activity level had returned to normal. However, there are caveats to this experimental design in that it is not clear what the proper controls in a study of early postpneumonectomy compensatory lung growth should be. Sham thoracotomy is traditionally used as the surgical control in these experiments. However, it is known that permanent lung collapse stimulates some compensatory lung growth in the mouse and rat (19, 48, 51). Furthermore, the mitotic index of the right lung in mice peaks at 2 days after left lung collapse (i.e., preceding the expected maximal weight increase period after pneumonectomy) (48). Although the data suggest that the small pneumothorax and partial collapse of the left lung induced by sham thoracotomy are irrelevant in relation to the long-term compensatory lung growth by *day 7*, the early physiological effects are unclear. In this context, the cDNA array was utilized as a screening system for analysis of a large number of candidate genes at an early time point in a complex physiological model, and Northern analysis was used with the additional sham thoracotomy control as the quantitative assessment of the transcription factors identified by the array screen.

Array Analysis

Numerous studies have demonstrated the power of the emerging cDNA array technology in assessing differential expression of genes in the complex biological processes involved in cancer, vascular biology, and response to viral infections. Most of these studies have focused on comparisons of cell lines or tumor tissue using homogeneous cell populations (33). The application of array technologies to whole organs in vivo is much more complex. In this regard, the scatterplot matrices of the phosphorimager densitometric measurements of each gene help to screen for "true positive" upregulated genes. The choice of appropriate reference standards for expression analysis has been debated (10, 49). Although no single gene represents a universal standard of constitutive expression, a linear plot in which the relative expressions of a group of commonly used control genes can be expressed as a composite value can then be used as a valid reference. The $\log_{10}$ plot of the phosphorimager values minus the average background signal of the blot itself facilitates screening because gene selection is based on a predetermined ratio of upregulated expression (60). We chose to limit our analysis to those transcription factors that demonstrated at least twofold upregulation (18, 46) in two independent experiments. This screen enabled the identification of six upregulated transcription factors, Egr-1, Nurr77, LRG-21, tristetraprolin, GKLF, and IκB-α, in the lungs of 2-h postpneumonectomy C57BL/6 mice.

Analysis of Upregulation Postpneumonectomy

Quantitative analyses of the autoradiographic hybridization signals for the six transcription factors of interest were compared among the three groups: unoperated controls, 2-h sham thoracotomy, and 2-h postpneumonectomy. Interestingly, although Northern analysis confirmed a significant upregulation at the mRNA level for each of these six genes in the postpneumonectomy group, in most cases, sham thoracotomy had a measurable, albeit smaller, effect on their ex-

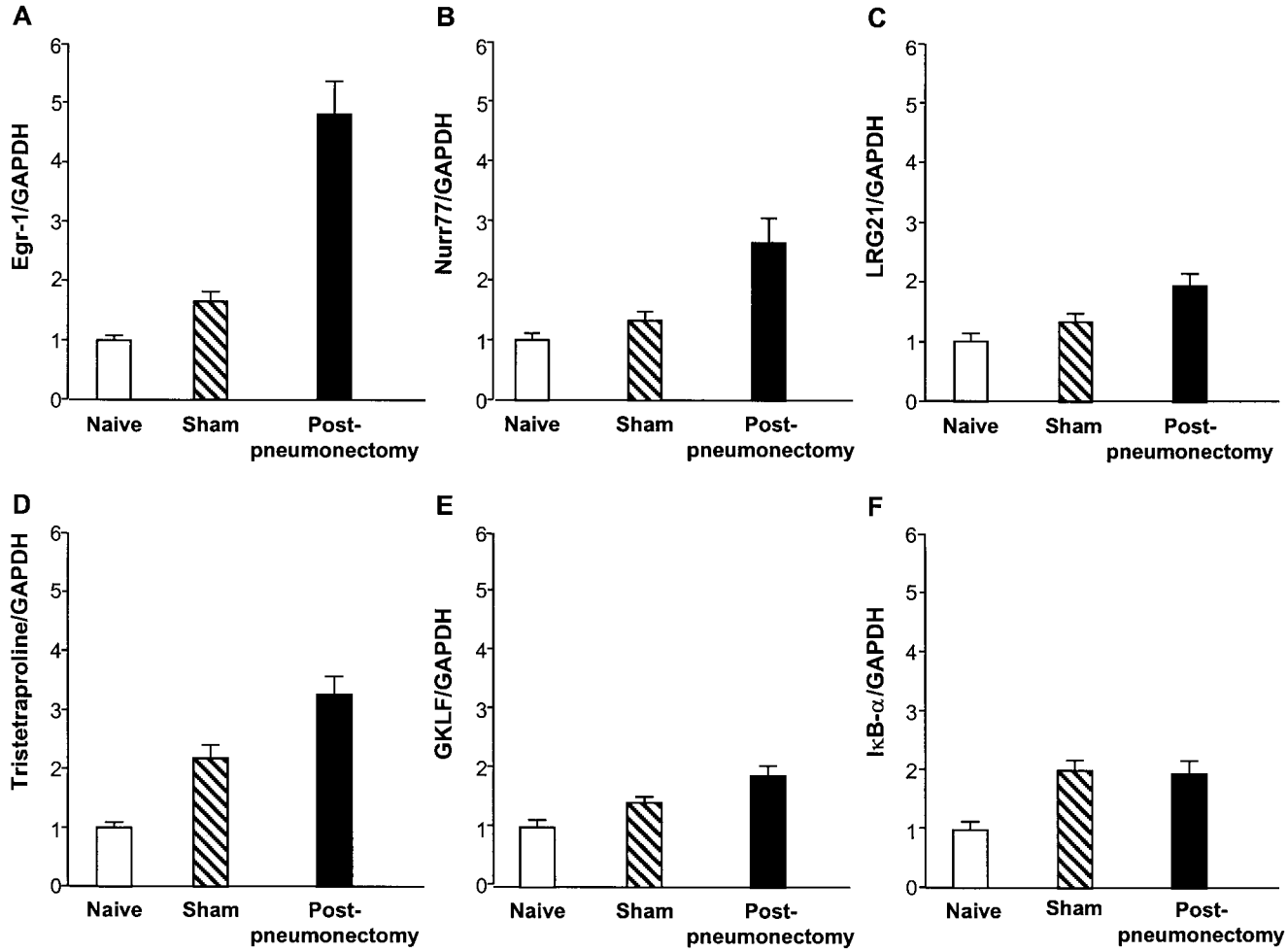

Fig. 6. Northern analysis quantification of the expression of upregulated mRNAs in postpneumonectomy lungs relative to unoperated naive and sham-operated controls. *A*: Egr-1. *B*: Nurr77. *C*: LRG-21. *D*: tristetraprolin. *E*: GKLF. *F*: IκB-α. For the lungs of each individual animal in each group, Northern analysis was used to quantify the expression of each mRNA of interest normalized to the expression of GAPDH. The phosphorimager densitometric value of each gene relative to GAPDH was calculated. Each bar represents the mean ± SE of mRNA expression of the specific gene of interest relative to that of the control gene GAPDH mRNA.

pression. It is possible that in the sham group, the short-term effects of thoracotomy and pneumothorax could partially mimic the acute effects of pneumonectomy with regard to blood flow and chest wall mechanical forces. In this context, one study has shown that disruption of pulmonary arterial blood flow after pneumonectomy modulates, but does not prevent, the compensatory growth response (29), suggesting a multifactorial etiology of postpneumonectomy lung growth.

Time-course analysis of the expression of each of the six transcription factors relative to GAPDH over the time period 2–24 h after left pneumonectomy or sham thoracotomy confirmed the transient nature of the upregulation at the mRNA level. The data are consistent with those of Gilbert and Rannels (13), which demonstrated transient upregulation of early response genes, c-*fos* and *junB*, in the very immediate postpneumonectomy period. Although the present study did not identify these particular genes as being upregulated, differences in experimental methodology and data analysis possibly affected which transcription factors were selected for further analysis. As stated above, candidate genes were strictly defined. In this context, c-*fos* and *junB* were not identified for further analysis. In the Atlas blot (Fig. 3), these and similar genes are located at *top left*. Although the present study did not specifically evaluate these genes, analysis of the phosphorimager data does not suggest that these genes are upregulated greater than twofold 2 h after pneumonectomy in C57BL/6 mice.

Specific Upregulated Transcription Factors Postpneumonectomy

Egr-1. Egr-1, also known as TIS8, krox-24, and NGFI-A, is a zinc finger transcription factor that is induced in a wide variety of cell types in response to diverse stimuli, including epithelial and endothelial injury and repair, hypoxia, and fluid shear stress (12, 21, 22, 59). Egr-1 expression has been linked to the expression of several important mediators and growth factors, including platelet-derived growth factor

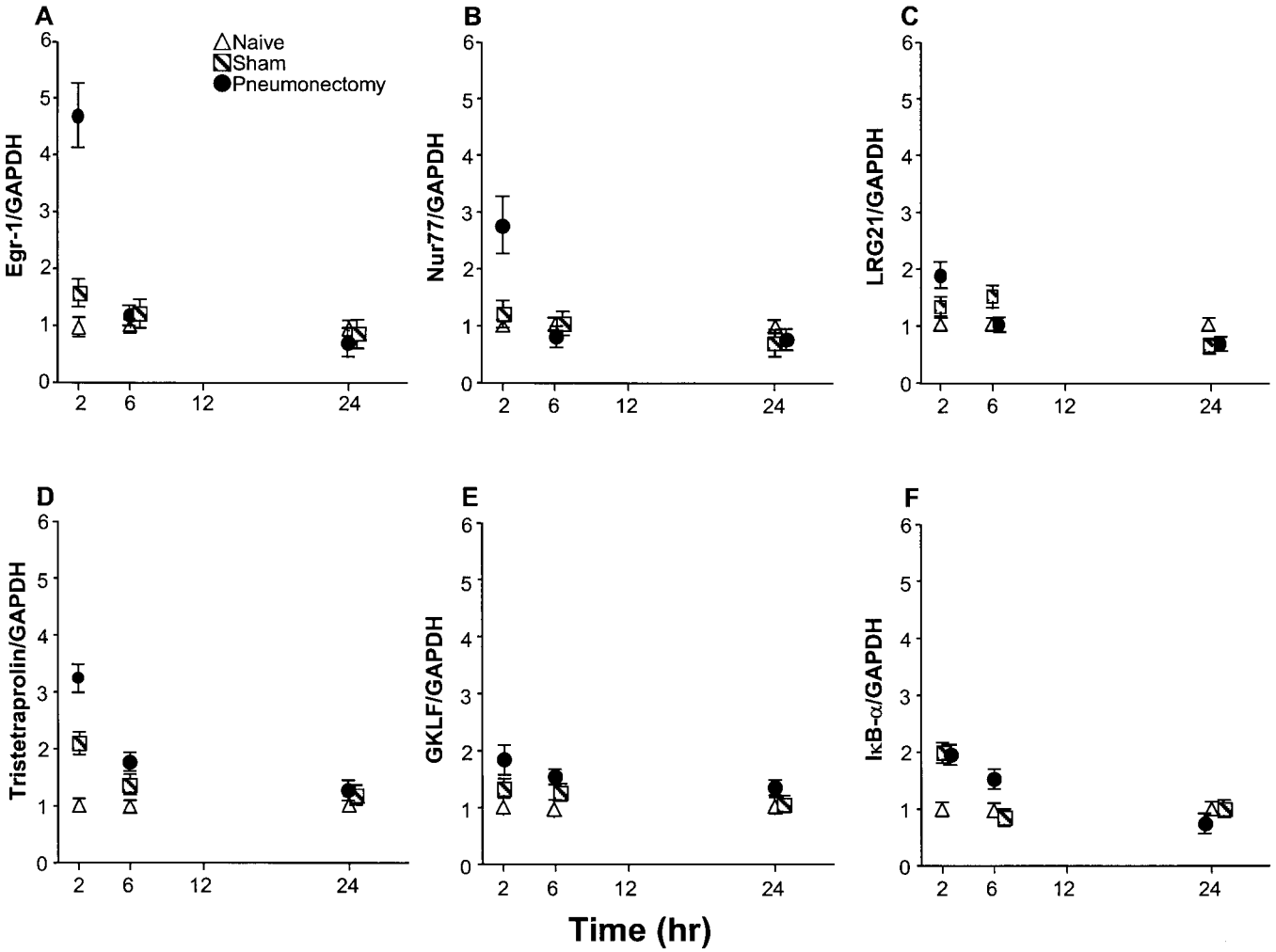

Fig. 7. Time-course analysis at 2, 6, and 24 h of the relative mRNA expression in the right lung after pneumonectomy for each of the identified transcription factors by Northern blotting compared with sham-operated control animals. *A*: Egr-1. *B*: Nurr77. *C*: LRG-21. *D*: tristetraprolin. *E*: GKLF. *F*: IκB-α. Phosphorimager quantification of expression was made for each individual animal in each group relative to the mRNA expression of each gene in the unoperated controls. Each point represents the mean ± SE of the mRNA expression of the specific gene of interest relative to the GAPDH control gene.

(PDGF) and fibroblast growth factor (FGF)-2, and the mitogen-activated protein (MAP), extracellular signal-regulated kinase (ERK), and c-*jun* NH_2-terminal kinase (JNK) kinase pathways (47). It has been extensively studied in models of endothelial cell injury by mechanical forces (12, 17, 21, 22, 42, 47, 59). In the lung, Egr-1 has been shown to be important for regulating macrophage differentiation in response to viral infections and inflammatory states (17). It is also induced in response to severe hypoxia and may drive tissue factor-mediated pulmonary fibrin deposition in mice (59). Interestingly, while hypoxia additionally enhances Egr-1 expression, hypoxia also enhances postpneumonectomy compensatory lung growth, whereas hyperoxia inhibits the process (42). Egr-1 is also upregulated in models of compensatory hepatic regeneration (6, 14). Although the present study does not address the mechanism of increased Egr-1 expression in postpneumonectomy compensatory lung growth, it is tempting to hypothesize that after left pneumonectomy, the remaining right lung suddenly receives at least 35% greater blood flow, leading to increased mechanical shear forces on the endothelium and subsequent induction of the Egr-1 pathway. However, Egr-1 cannot be a critical gene that switches on the entire process of lung growth, since Egr-1(−/−) knockout mice survive to adulthood and are not known to have abnormalities in lung structure (26, 27).

Nurr77. Nurr77 (also known as NGFI-B, N10, TIS1, and NAK-1) is a member of the steroid/thyroid hormone receptor family of transcription factors (16). It is rapidly and transiently induced in response to growth and differentiation signals, for example, in neuronal cells in response to nerve growth factor (30). It has also been shown to be induced in response to partial hepatectomy in liver regeneration models (40). It is expressed in the lung and lung cancer cell lines (28, 55), but its function in the normal lung is not known.

LRG-21. LRG-21 is an immediate-early gene with sequences containing basic and leucine zipper regions characteristic of the c-*fos* and c-*jun* family of transcription factors (9). It is rapidly induced in macrophages in response to a variety of inflammatory stimuli, including lipopolysaccharide, interleukin-4, bacillus Calmette-Guerin, and interferon-γ. Other than its role in macrophages and its probable role in response to stress, inflammation, and infection, LRG-21 does not have a described function in the lung parenchyma.

Tristetraprolin. Tristetraprolin (TTP; also known as G0S24, Nup475, and TIS11) is another immediate-early gene and the prototype member of a family of zinc finger transcription factors (25). In normal tissues, TTP expression is widely distributed, with high levels in the lung, lymph nodes, and spleen. In fibroblasts, it is known to be rapidly and transiently (within 2 h) induced in response to a variety of stimuli, including serum, polypeptide growth factors, and phorbol 12-myristate 13-acetate (53, 54). It has been shown to function as an antagonist to tumor necrosis factor (TNF)-α in that it destabilizes its mRNA (24). Thus at least one of its functions appears to involve an autoregulatory feedback against TNF-α expression in the setting of stress or inflammation. These data may be relevant to the observation that sham thoracotomy itself induced a significant upregulation of TTP mRNA relative to unoperated controls.

GKLF. GKLF is a member of a subset of interrelated zinc finger transcription factors with similarity to the *Drosophila* segmentation gene *Krüppel* (44, 45). Interestingly, GKLF has been described as a "growth arrest" gene in that its expression is markedly downregulated in rapidly proliferating NIH/3T3 cells, but the exact role of GKLF in the cell cycle or in differentiation remains unknown. It is known to be expressed in the lung, but its role in the lung is unknown (44). The related lung-enriched Krüppel-like factor has been described and is known to be developmentally regulated (1). Interestingly, it was not differentially expressed on the Atlas blot.

IκB-α. NF-κB represents a transcription factor composed of a family of proteins that form hetero- and homodimers and bind DNA to influence transient transcription of many genes in response to diverse pathological and physiological stimuli, including immunity, host defense, and stress response (2). In its inactive form, NF-κB is located in the cytosol bound by its primary inhibitor, IκB-α. On relevant cellular stimulation, IκB-α is degraded, which allows for translocation of NF-κB to the nucleus. IκB-α has been shown to be induced in response to cellular stress response, attenuating NF-κB nuclear translocation and ultimately providing an autoregulatory mechanism during inflammatory states (57, 58). The NF-κB system has been studied in lung disease (38), with particular focus on models of acute lung injury and the acute respiratory distress syndrome (ARDS) (3, 31, 32, 41, 43). In acute lung injury in humans, there is increased nuclear translocation of NF-κB in lung mononuclear cells (31, 41). Normal human volunteers have minimal NF-κB activation (11). In a murine model of acute lung injury induced by hemorrhage, cytoplasmic and nuclear IκB-α proteins were transiently and maximally increased by 60 min in cultured lung mononuclear cells (32). Relative to the present study, pneumonectomy induced a variable but small amount of hemorrhage during lung removal, whereas the amount of blood loss in the sham-treated mice was minimal. In addition, alveolar macrophages subjected to mechanical ventilation show activation of the NF-κB system (37). Nevertheless, IκB-α mRNA was upregulated equally in both the pneumonectomy and sham groups, suggesting that the response to surgery and anesthesia but not blood loss accounts for the nonspecific upregulation of this gene.

Initiation of Lung Growth

The use of array technology has enabled the identification of several transcription factors that are candidates for contributing to the growth of the lung after pneumonectomy. The fact that sham thoracotomy also variably induced the expression of some of these genes at the mRNA level does not invalidate these results, since the inductive mechanisms governing early postpneumonectomy lung growth are undoubtedly linked to mechanical factors inducing a complex array of molecular events. Each of the six transcription factors identified by this analysis has known important roles in vascular biology, embryology and development, and stress response. Future studies will focus on the individual genes and attempt to define their roles in the complex physiological events occurring during postpneumonectomy compensatory lung growth.

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