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Chronic cardiac rejection: Identification of five upregulated genes in transplanted hearts by differential mRNA display

(gene expression/transplant arteriosclerosis/cardiac transplantation/polymerase chain reaction)

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ABSTRACT **Transplant arteriosclerosis, the major manifestation of chronic rejection, develops after allogeneic (Lewis to F344) but not syngeneic (Lewis to Lewis) rat cardiac transplantation. To identify transcriptionally regulated mediators associated with chronic cardiac rejection, we adapted the differential mRNA display technique for *in vivo* transplant specimens. Gene transcript patterns in four allogeneic hearts showing early signs of chronic rejection were compared with those in two syngeneic hearts exposed to the same surgical procedure but histologically normal. Twelve differentially expressed cDNA bands were identified. We improved the probability of isolating one or more allograft-specific cDNAs from a single display band by first using recovered and reamplified PCR products as probes in RNA blot analysis. cDNA fragments cloned from individual bands were then used in a second RNA blot analysis, which allowed for the correlation of specific mRNA transcripts with cDNA clones. Five cDNA clones produced time-dependent, allograft-specific hybridization. Sequence analysis demonstrated that two of these cDNAs corresponded to unknown genes, whereas the other three represented known genes not previously associated with chronic rejection. The latter group included the macrophage lectin specific for galactose/*N*-acetylgalactosamine (a cell-surface receptor), the nuclear P1 gene (a homologue of a yeast replication protein), and a ubiquitin-like gene. Our application of the differential display technique allowed the direct identification of potential mediators under *in vivo* conditions that preserve the environment of the disease process—including infiltrating cell populations critical to the inflammatory response.**

For most genes, expression is regulated at the level of transcription; therefore, the evaluation of gene transcripts has become an important tool in the study of normal and pathologic processes. Conventional measurements of mRNA transcript levels are usually confined to selected genes of interest and often require information about the gene sequence. In contrast, PCR-based differential display techniques circumvent this constraint by allowing comparison of gene expression patterns between two cell populations (1) or between various murine organs (2). One of the principal advantages of differential display is that it permits the simultaneous identification of genes that are upregulated as well as genes that are downregulated. Thus differential display has the potential to identify a spectrum of molecular factors—known and unknown—that are differentially regulated in cells under various conditions.

Little is known about molecular factors involved in the pathogenesis of chronic cardiac rejection. In contrast with acute rejection, this multicellular process results in an obliterative neointimal thickening in the vessels of the transplanted heart that limits transplant survival (3, 4). Studies of the process in humans have been restricted by the limited availability of tissue for analysis. Clinical specimens are heterogeneous in their degree of chronic rejection, their extent of superimposed disease processes, and the period between the time they are obtained and the time of transplantation. Also, transplanted hearts obtained at autopsy are not suitable for analysis (which requires viable tissue), and the utility of endomyocardial biopsy specimens is limited by their small size. Moreover, the restricted extent of arteriosclerotic lesions that follow transplantation suggests that the process is locally regulated; thus, studies measuring systemic levels of factors implicated in chronic rejection may not accurately reflect levels within the graft (5).

A heterotopic rat cardiac transplantation model (which results in long-term graft survival) has provided insight into the cellular development of chronic cardiac rejection (6, 7). Monocytes/macrophages accumulate in the first stage (days 7–30) of arteriosclerotic thickening. In the intermediate stage (days 30–75) both macrophages and smooth muscle cells accumulate, and in the later, more obliterative stage (after 75 days) smooth muscle cells predominate. In previous studies we used reverse transcription PCR to measure gene transcript levels for selected cytokine factors, and we showed that interferon γ and monocyte chemoattractant protein 1 transcript levels were upregulated in cardiac allografts in a specific and local manner (ref. 8 and M.E.R., A. F. Wallace, N. E. S. Sibinga, L.R.W., and M.J.K., unpublished data). However, chronic rejection is most likely mediated by a variety of known as well as unknown factors. Therefore we performed the present study to determine whether differential mRNA display could be used to identify unknown mediators or known mediators not previously implicated in chronic cardiac rejection. We adapted the technique to the evaluation of gene expression patterns directly in diseased tissues by comparing mRNA profiles in four arteriosclerotic rat hearts after allogeneic transplantation with those in two control hearts subjected to syngeneic transplantation. Using 27 primer combinations, we identified two unknown and three known genes whose expression is upregulated with time in hearts undergoing chronic rejection.

MATERIALS AND METHODS

RNA Isolation and RNA Blot Analysis. Heterotopic abdominal cardiac transplantations were performed and samples were collected as described (8, 9). For allogeneic transplantations we used Lewis rats as graft donors and F344 rats as recipients. This strain combination generates grafts that survive beyond 7 days, the point after which rejection would be considered chronic. The early signs of chronic rejection—macrophage infiltration and adhesion to luminal surfaces—

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were confirmed by histologic analysis as described (7, 9). Syngeneic control procedures (Lewis to Lewis) were performed to assess the contribution of surgical manipulation to the inflammatory response. Hearts were harvested at various points after transplantation and quick-frozen in liquid nitrogen. Total cellular RNA was extracted from heart tissue with RNAzol B (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. Samples of total RNA (20 μg) were fractionated in 2.2 M formaldehyde/1% agarose gels and transferred onto nylon-supported nitrocellulose (Micron Separations, Westboro, MA) by standard capillary blotting techniques. Specific probes were generated by labeling reamplified or cloned cDNA fragments with [α-^{32}P]dCTP (random prime DNA labeling kit, Boehringer Mannheim). After hybridization at 42°C and high-stringency washes at 60°C in 0.3 M NaCl/0.03 M trisodium citrate, pH 7/0.1% SDS, the blots were exposed to Kodak X-omat AR film for 3–7 days with intensifying screens. Hybridization with a partial cDNA fragment of the rat homologue of 36B4 (acidic ribosomal phosphoprotein P0) was used as a loading control (10).

Differential mRNA Display. Differential mRNA display analysis was carried out as described (1, 11), except that *in vivo* rather than *in vitro* samples were used and six rather than two samples were compared simultaneously. We studied total RNAs obtained from four allografts (two each harvested at days 7 and 14) and two syngrafts (harvested at day 14). The cDNA and PCR reactions were modified as follows. Total RNA (0.5 μg) was reverse transcribed in a 50-μl reaction mixture with Superscript reverse transcriptase (GIBCO/BRL) and the degenerate oligo(dT) primer (Genosys, The Woodlands, TX) T_{12}VC or T_{12}VA (where V represented a mixture of dG, dA, or dC). Control reactions were performed in the absence of reverse transcriptase. The cDNAs were then amplified by PCR in the presence of [α-[^{35}S]thio]dATP on a Perkin–Elmer 9600 thermal cycler, and control studies were performed in which water was substituted for cDNA. The reaction mixtures (20 μl) included arbitrary decanucleotides (kit A, Operon Technologies, Alameda, CA) as 5′ primers and T_{12}VC or T_{12}VA as 3′ primers. Parameters for the 40-cycle PCR were as follows: denaturation at 94°C for 15 sec, annealing at 40°C for 60 sec, and extension at 70°C for 20 sec. Radiolabeled PCR amplification products were analyzed by electrophoresis in denaturing 6% polyacrylamide gels. We observed a variability of 5–20% in the number and intensity of bands among given samples on repeated PCR analyses, as well as among different allogeneic or syngeneic samples in the same PCR analysis. To confirm the reproducibility of amplification for selected bands, we repeated the reactions at least three times with different preparations of cDNA. Differentially upregulated bands were defined as those that were consistently present in all four allogeneic samples and absent from both syngeneic samples. Differentially downregulated bands were defined as those present only in syngeneic samples. PCR product bands of interest were recovered from sequencing gels and reamplified in a 40-cycle PCR (80-μl mixture) in the absence of isotope. Reamplified cDNAs ranging from 100 to 500 bp were used for cloning into plasmid vectors and as templates for random priming.

TA Cloning and Sequence Analysis. Reamplified cDNA fragments were cloned into the plasmid vector pCRII using the TA cloning kit (Invitrogen). Inserted cDNAs were isolated, radiolabeled, and used as probes in RNA blot analysis. cDNA fragments that generated a specific hybridization pattern on RNA blots were sequenced in both directions (Sequenase kit 2.0, United States Biochemical). The nucleotide sequences obtained were compared with known sequences by searching the GenBank and EMBL data bases (March 1994) with the FASTA program [Genetics Computer Group (Madison, WI) software].

RESULTS

Differential mRNA Display. To identify transcriptionally regulated genes potentially involved in the early stages of chronic rejection, we compared differential mRNA display patterns for hearts from syngeneic transplantations with those for hearts from allogeneic transplantations. Syngeneic hearts were normal on histologic examination, whereas 7- and 14-day allogeneic hearts showed luminal monocyte adhesion and infiltration without intimal thickening (as described previously; ref. 9). We performed PCR amplifications with 27 primer combinations on all six samples and identified 12 PCR products, designated bands 1–12, that were differentially expressed between allogeneic and syngeneic tissue. Fig. 1 shows PCR amplifications obtained with three separate primer combinations. Indicated are four representative PCR products (bands 1, 2, 11, and 12) that were reproducibly present in the allogeneic samples (lanes 3–6) but not in the syngeneic samples (lanes 1 and 2) in each of the three analyses.

RNA Blot Analysis with PCR-Amplified Fragments. To confirm the gene regulation patterns observed in the differential display study, we recovered the 12 selected bands, reamplified them, and used them to probe RNA blots prepared with RNAs from syn- and allogeneic transplantations. When used as probes, 4 of the 12 PCR-amplified fragments (bands 1, 2, 11, and 12) generated hybridization patterns that reproduced the allograft-specific increase in expression (Fig. 2*A*, lanes 3–6). Unexpectedly, each of these 4 probes generated two hybridization signals of different sizes. The two signals identified by the band-1 and -11 probes were both specifically present in allografted tissues (lanes 3–6, arrows) and absent from syngrafted tissues (lanes 1 and 2). In contrast, the band-2 and -12 probes each generated one allograft-specific signal (arrows) reproducing the differential

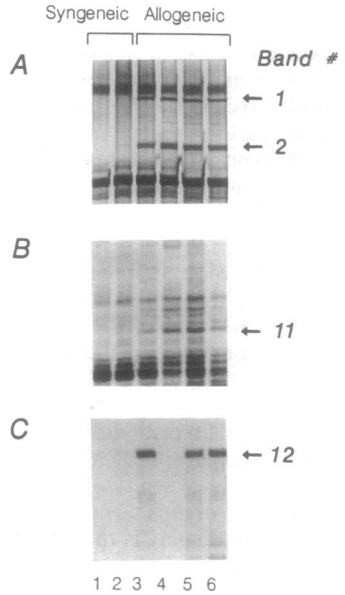

FIG. 1. Differential display comparing RNAs from syngeneic and allogeneic hearts. Total RNA was extracted from hearts 14 days (lanes 1 and 2) after syngeneic transplantation and 7 days (lanes 3 and 4) and 14 days (lanes 5 and 6) after allogeneic transplantation and subjected to differential mRNA display analysis. Autoradiograms of amplified [α-[^{35}S]thio]dATP-labeled PCR products (after electrophoresis in 6% polyacrylamide gels) are shown for three different primer combinations (*A–C*) that identified four distinct fragments (arrows) upregulated in the allogeneic group. Primer combinations included T_{12}VC as 3′ primer for all reactions and various 5′ primers: *A*, OPA-16 (AGCCAGCGAA); *B*, OPA-04 (AATCGGGCTG); and *C*, OPA-14 (TCTGTGCTGG). Lane 4 in *C* shows a PCR that failed.

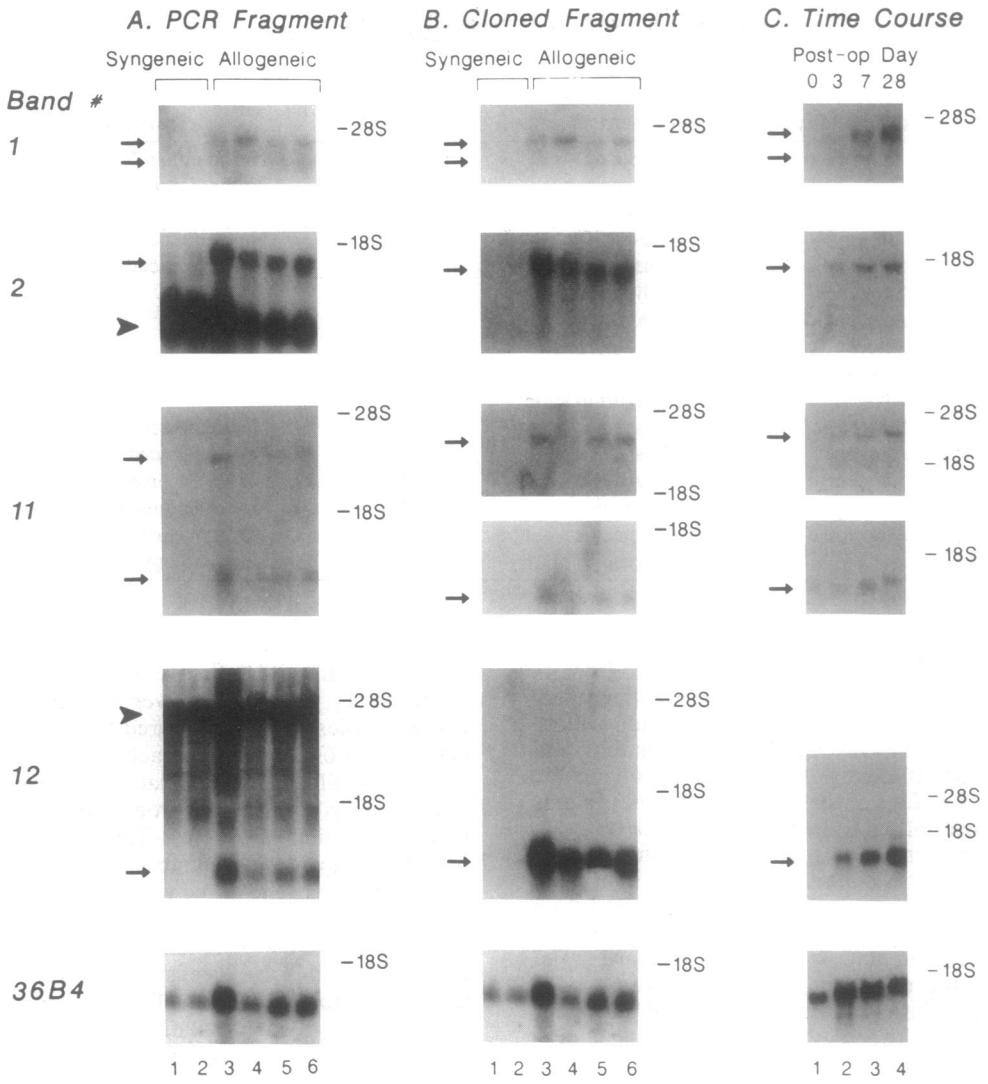

FIG. 2. RNA (Northern) blot analysis confirming allograft-specific gene induction for differential display bands 1, 2, 11, and 12. (*A* and *B*) Total RNA (20 μg per lane) obtained from day 14 (lanes 1 and 2) syngeneic and day 7 (lanes 3 and 4) and day 14 (lanes 5 and 6) allogeneic transplantations was hybridized with cDNA probes generated by PCR reamplification of bands recovered from differential display gels (*A*) or cloned cDNA fragments (*B*). Arrows indicate allograft-specific hybridization patterns. Arrowheads indicate hybridization in all six lanes (which was considered nonspecific). (*C*) Total RNA obtained from allogeneic transplantations at days 0, 3, 7, and 28 was hybridized with the cloned cDNA fragments as in *B*. For all five genes identified by differential display, expression increased with time after transplantation. (*A–C*) RNA loading was evaluated by reprobing the same blot with the rat 36B4 homologue (*Bottom*). Positions of 18S and 28S rRNA are indicated as size markers.

display pattern, as well as a second signal present in all six lanes (arrowheads) that did not reproduce the differential display pattern. Three of the 12 reamplified PCR fragments hybridized nonspecifically to all six lanes and were not studied further. The remaining 5 did not detect any transcripts (data not shown). Such transcripts may not have been detected because their levels were below the sensitivity of the RNA blot analysis. RNA loading in all six lanes was confirmed by hybridization with the ribosomal reference gene 36B4 (10).

RNA Blot Analysis with Cloned Fragments. The PCR products that generated one or more allograft-specific hybridization patterns were then cloned and used as hybridization probes in RNA blot analysis to identify single clones corresponding to specific mRNA transcripts (Fig. 2*B* and Table 1). For bands 2 and 12, individual cDNA clones were identified that produced hybridizations in an allograft-specific fashion. Identification of individual clones was more arduous for the bands that had generated two allograft-specific signals in the initial RNA blot analysis. Two separate mechanisms account for the transcripts of two sizes observed for band 1 compared with band 11. For band 1, an individual cDNA clone generated two faint hybridization signals of 3.5 kb and 1.5 kb specifically in the allograft samples (lanes 3–6). This suggests that the two mRNAs were generated by alternative splicing of a common mRNA precursor, by a gene duplication event, or, less likely, by a common regulatory pattern for genes that share some homology. For band 11, however, two independent cDNA clones with allograft-specific regulation were isolated: one hybridizing to a 1.0-kb transcript and the other to a 3.5-kb transcript. These two distinct clones hybridizing to transcripts of two sizes demonstrate that PCR-amplified products from a display band can contain a number of distinct cDNA fragments derived from different genes (Table 1). Thus, in the initial RNA blot screen, the PCR-reamplified fragment (which may contain a mixture of PCR products) is more likely to identify differentially regulated transcripts than are individual cDNA clones.

Time-Dependent Increase in Gene Transcript Levels after Allogeneic Transplantation. We also used the cloned frag-

Table 1. Analysis of cDNA fragments identified by differential mRNA display

Band	Expression pattern: Differential display	Expression pattern: Northern analysis	Transcript size, kb	Sequence homology
1	Allogeneic	Allogeneic	3.5	No homology*
		Allogeneic	1.5	No homology*
2	Allogeneic	Allogeneic	1.4	Rat macrophage Gal/GalNAc lectin
		Nonspecific	1.0	Not sequenced
3	Allogeneic	Nonspecific	1.8	Not sequenced
4	Syngeneic	No hybridization		
5	Allogeneic	No hybridization		
6	Allogeneic	No hybridization		
7	Allogeneic	No hybridization		
8	Allogeneic	No hybridization		
9	Syngeneic	Nonspecific	1.0	Not sequenced
10	Allogeneic	Nonspecific	1.6	Not sequenced
11	Allogeneic	Allogeneic	3.5	Mouse P1 protein
		Allogeneic	1.0	Mouse ubiquitin-like protein
12	Allogeneic	Nonspecific	5.0	Not sequenced
		Allogeneic	0.7	No homology

*Single cDNA clone hybridized to transcripts of two sizes.

ments in RNA blot analyses to evaluate changes in gene transcript levels over time (Fig. 2*C*). We analyzed hearts undergoing chronic rejection as early as day 3, when macrophage infiltration begins, at day 7, when infiltration is more evident and adhesion to the vascular lumen is apparent, and as late as day 28, when the signs of chronic rejection include intimal thickening. For four of the five cDNA clones [bands 1, 2, 11 (3.5-kb transcript) and 12], no signal was detected in day 0 hearts (which were harvested but not transplanted). Faint hybridization was visible at day 3, and it became stronger at days 7 and 28. For the fifth clone (band 11, 1.0-kb transcript), hybridization was faint in day 0 and day 3 hearts but more pronounced in day 7 and day 28 hearts. The experiments show that transcript levels for all five clones increased with time. These patterns of early and persistent gene induction would be expected given the ongoing allogeneic stimulation associated with chronic rejection.

Sequence Homology. Cloned cDNA fragments that generated allograft-specific hybridization patterns in the RNA blot analysis were partially sequenced, and preliminary homology searches were performed. The results are summarized in Table 1. The cDNA fragment from band 2 was found to be highly homologous to rat macrophage lectin specific for galactose/*N*-acetylgalactosamine (Gal/GalNAc). The 383-bp fragment was 98% identical to bases 975–1357 of the published lectin sequence (12). This region includes 114 bp of open reading frame, as well as 3′ untranslated sequence. Homologies with two distinct genes were identified for the two independent clones associated with band 11. The cDNA fragment (110 bp) that hybridized to the smaller mRNA transcript (1.0 kb) was 79% identical to the 3′ untranslated region of a partial cDNA sequence obtained from a mouse ubiquitin-like gene (13). The cloned fragment (119 bp) that hybridized to the larger transcript (3.5 kb) was 92% identical to a partial cDNA sequence of the mouse nuclear P1 gene (14). Unexpectedly, the homologous region was located within the open reading frame (bases 1–120) and not at the 3′ end. Therefore, in this instance the 3′ primer of the initial PCR hybridized to an internal sequence. To date we have found no significant homology with any published gene for the sequences obtained from the band-1 and -12 cDNA fragments, suggesting that they represent previously unknown genes associated with chronic cardiac rejection.

DISCUSSION

Our study demonstrates that the differential mRNA display technique can be adapted to identify mediators associated with a complex, multicellular disease process such as chronic rejection. Differential display has been used to study breast cancer (1, 11, 15, 16). However, in contrast with the breast-cancer studies, which compared two populations of *in vitro* cell lines at once, our approach involved comparing whole tissue from allogeneic transplantations (where chronic rejection develops) with tissue from syngeneic transplantations (where rejection is absent). This approach preserved the pathophysiologic environment associated with the chronic disease process. The tissue we studied included a mixture of both resident and infiltrating cells, as well as the complex network of regulatory stimuli that may have been impossible to reproduce in isolated cells *in vitro* (17). Also, because our analysis compared a series of six transplanted hearts simultaneously, we avoided isolating factors that might have been related to a single animal or procedure rather than to the disease itself. Most importantly, we performed each differential display analysis at least three times to reduce nonspecific (background) PCR signal interference, and we restricted the selection of cDNA bands for further study to those that reproduced the regulation pattern of the first differential display analysis in at least three analyses.

Using 27 primer combinations we identified 12 differential display cDNA bands that were reproducibly up- or down-regulated in allogeneic hearts. For 4 of the 12 bands, this allograft-specific regulation was reproduced on RNA blot analysis. We identified from these 4 bands two unknown genes and three known genes not previously implicated in chronic rejection. Finally, we showed that the increase in transcript levels for these five genes was time dependent, implying a sustained gene induction in the early phase of chronic rejection.

One of our goals in undertaking this study was to identify mediators that might be selective for or specific to chronic rejection. The two genes whose partial nucleotide sequences showed no homology to genes in public data bases may represent such mediators. Having demonstrated a specific association between these genes and chronic rejection, we can now gain insight into their precise roles by isolating and analyzing their full-length cDNAs, identifying the specific cell types that express them (or their products) and studying their function.

The three upregulated genes with identifiable homologies correspond to the macrophage Gal/GalNAc lectin gene, the nuclear P1 gene, and a ubiquitin-like gene. The link between the macrophage lectin gene and chronic rejection is especially

interesting because the factors responsible for macrophage accumulation in the early phase of the process are not known. Lectins are cell-surface molecules that mediate cell–cell interactions by recognizing specific sugar molecules on adjacent cells (18). The murine Gal/GalNAc-specific lectin was identified by immunofluorescence on thioglycolate-elicited and OK-432-activated macrophages (OK-432 is a streptococcal antitumor preparation) but not on unstimulated or resident macrophages (19). Our finding that macrophage lectin transcript levels are higher only in allogeneic transplanted heart tissue raises the possibility that the lectin plays a role in the localization or even activation of macrophages in transplant arteriosclerosis.

The mouse P1 protein, a homologue of yeast MCM3 (minichromosome mutant), plays a role in the initiation of DNA replication in association with DNA polymerase α primase (14). The identification of elevated transcript levels for the P1 gene in cardiac allografts compared with syngrafts suggests the presence of replicating cells at early points in chronic rejection. Localizing the specific cell type that expresses P1 gene transcripts (or protein) by *in situ* hybridization (or immunohistochemistry) may help us understand early proliferative processes in chronic rejection.

The third known gene upregulated in cardiac allografts is homologous to the 3′ region of a murine ubiquitin sequence (13). As its name implies, ubiquitin is expressed in all eukaryotic cells. Our findings indicate that ubiquitin gene transcripts may be upregulated in allogeneic tissue. Although ubiquitin is involved in a wide variety of regulatory functions within the cell, its role in protein degradation is best understood. In that process ubiquitin is covalently attached to a specific protein target which is then recognized and degraded as part of normal protein turnover (20). However, the induction of ubiquitin is also part of the cellular response to stress, damage, or injury (21). Although ubiquitin's specific role in chronic cardiac rejection is not clear, it is possible that ubiquitin is involved in the response to immune injury thought to initiate allograft arteriosclerosis.

With the identification of these five candidate mediators of chronic rejection, we demonstrate the potential of differential mRNA display analysis to provide insight into molecular factors associated with complex multicellular processes. In the case of chronic rejection, which affects the donor organ only and spares host organs, differential mRNA display allowed us to examine the transplanted heart as well as its infiltrating cell populations. Given that inflammatory cells are often activated in a manner specific to their microenvironment, the power of this technique resides in its preservation of infiltrating cells and the complex network of regulatory influences in the tissue under investigation. *In vitro* systems investigating single cell types cannot reproduce the spectrum of interactions present in diseased tissue *in vivo* because they lack the counterregulatory effects of neighboring cells. The differentially regulated factors we have identified are therefore more likely to be of direct clinical relevance. Finally, our differential display approach allows the identification of candidate factors that may be beyond the scope of established theories of chronic rejection.

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1. Liang, P. & Pardee, A. B. (1992) *Science* **257**, 967–971.
2. Welsh, J., Chada, K., Dalal, S. S., Cheng, R., Ralph, D. & McClelland, M. (1992) *Nucleic Acids Res.* **20**, 4965–4970.
3. Schoen, F. J. & Libby, P. (1991) *Trends Cardiovasc. Med.* **1**, 216–223.
4. Cramer, D. V. (1993) *Graft Arteriosclerosis in Heart Transplantation* (Landes, Austin, TX).
5. Fyfe, A., Daly, P., Galligan, L., Pirc, L., Feindel, C. & Cardella, C. (1993) *J. Am. Coll. Cardiol.* **21**, 171–176.
6. Cramer, D. V., Wu, G. D., Chapman, F. A., Cajulis, E., Wang, H. K. & Makowka, L. (1992) *J. Heart Lung Transplant.* 458–466.
7. Adams, D. H., Wyner, L. R. & Karnovsky, M. J. (1993) *Transplantation* **56**, 794–799.
8. Russell, M. E., Adams, D. J., Wyner, L., Halnon, N. J., Yamashita, Y. & Karnovsky, M. J. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6086–6090.
9. Adams, D. H., Tilney, N. L., Collins, J. J. & Karnovsky, M. J. (1992) *Transplantation* **53**, 1115–1119.
10. Laborda, J. (1991) *Nucleic Acids Res.* **19**, 3998.
11. Liang, P., Averboukh, L. & Pardee, A. B. (1993) *Nucleic Acids Res.* **21**, 3269–3275.
12. Ii, M., Kurata, H., Itoh, N., Yamashina, I. & Kawasaki, T. (1990) *J. Biol. Chem.* **265**, 11295–11298.
13. Kumar, S., Tomooka, Y. & Noda, M. (1992) *Biochem. Biophys. Res. Commun.* **185**, 1155–1161.
14. Thoemmes, P., Fett, R., Schray, B., Burkhart, R., Barnes, M., Kennedy, C., Brown, N. C. & Knippers, R. (1992) *Nucleic Acids Res.* **20**, 1069–1074.
15. Liang, P., Averboukh, L., Keyomarsi, K., Sager, R. & Pardee, A. B. (1992) *Cancer Res.* **52**, 6966–6968.
16. Sager, R., Anisowicz, A., Neveu, M., Liang, P. & Sotiropoulou, G. (1993) *FASEB J.* **7**, 964–970.
17. Liaw, L. & Schwartz, S. M. (1993) *Arterioscler. Thromb.* **13**, 985–993.
18. Sharon, N. & Lis, H. (1989) *Science* **246**, 227–234.
19. Oda, S., Sato, M., Toyoshima, S. & Osawa, T. (1988) *J. Biochem. (Tokyo)* **104**, 600–605.
20. Hershko, A. & Ciechanover, A. (1992) *Annu. Rev. Biochem.* **61**, 761–807.
21. Mayer, R. J., Arnold, J., Laszlo, L., Landon, M. & Lowe, J. (1991) *Biochim. Biophys. Acta* **1089**, 141–151.