Extinction of the Tumor Necrosis Factor Locus, and of Genes Encoding the Lipopolysaccharide Signaling Pathway

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Summary

The tumor necrosis factor (TNF-α or TNF) gene is activated by both lipopolysaccharide (LPS) and cycloheximide in RAW 264.7 macrophages, whereas neither stimulus activates the gene in 3T3 fibroblasts. Moreover, the pattern of CG methylation within the TNF gene is readily distinguishable in DNA derived from cells of these two types. These findings would suggest that the TNF gene has been rendered inaccessible to transcription in the 3T3 cell environment. When RAW 264.7 cells are fused with 3T3 cells, an immortal pentaploid hybrid results. In the hybrid cell, all three TNF genes contributed by the RAW 264.7 cell parent become highly methylated according to the pattern observed in the 3T3 cell parent. Permanently transfected chloramphenicol acetyl transferase (CAT) reporter constructs, bearing 2.2 kb of upstream sequence (including the entire TNF promoter and 5'-untranslated region [UTR]) as well as 1.0 kb of downstream sequence (including the entire TNF 3'-UTR and termination sequence), are accessible in both RAW 264.7 cells and 3T3 cells, but are silenced in transition from the RAW 264.7 cell to the hybrid cell environment. Moreover, the endotoxin signaling pathway is abrogated, as assessed by transient transfection of hybrid cells with LPS-responsive CAT reporter constructs. It would therefore appear that the fusion of 3T3 cells and RAW 264.7 cells activates a system that silences the TNF gene, as well as the LPS signaling pathway. This system may operate to determine TNF gene accessibility and LPS responsiveness in the course of cell differentiation. The DNA sequences targeted within the TNF gene are included in the CAT reporter construct; therefore, the silencing element has been circumscribed to a region of DNA 3.2 kb in length.

Previous work has revealed that expression of the TNF gene is regulated both at transcriptional and at translational levels in macrophages (1). The duality of control mechanisms perhaps assures that induction at the protein level is of a very high order or, stated differently, that little or no TNF protein is made by cells in the absence of an activating stimulus.

However, additional regulatory mechanisms, chiefly of a suppressive character (2), must surely be set in place during cell differentiation. So much is evident from the fact that transfected copies of TNF promoter are well utilized in most (and probably all) cells, whereas the TNF locus itself is absolutely silent (3); comparatively few cell types are capable of expressing the TNF mRNA or protein (4–10). Among cells that are capable of expressing the TNF gene, the macrophage is unique, insofar as it is capable of secreting ~1,000 times more TNF in response to LPS (11–14) than any other cell type. One might therefore suppose that the TNF gene is highly accessible in macrophages, or that macrophages are endowed with factors that permit high-level expression of this gene.

To examine the accessibility of the TNF gene in a type of cell that does not normally express TNF, and to analyze the suppressive mechanisms that maintain the gene in a permanently inactive state, we investigated the behavior of the TNF gene in 3T3 cells. TNF is not normally synthesized by 3T3 cells, in response to LPS or any other known stimulus (our unpublished observations), and therefore, these cells may serve as a model system in which to study repression of the TNF gene. RAW 264.7 macrophages maintain the TNF gene in an accessible form, and display an intact LPS signal transduction pathway (14–16). We sought to analyze differences between the RAW 264.7 and 3T3 phenotypes through studies of TNF gene structure, gene accessibility, and LPS signaling competence. Because we felt that 3T3 cells might lack any means of responding to LPS (e.g., might lack a receptor for LPS, or any number of proteins required for signal transduction), a more direct means of stimulating the TNF gene was used in these cells. Cycloheximide was used to test accessibility of the TNF gene (and certain reporter genes), since protein synthesis inhibitors have been shown to suppress the biosynthesis of labile repressors that block TNF gene tran-

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/05/1383/08 $2.00
Volume 177 May 1993 1383–1390

scription, eliciting "superinduction" of a type independent of endotoxin signal transduction pathways (17).

We sought to determine whether the mechanism responsible for suppression of LPS-induced TNF synthesis in 3T3 cells was dominant or recessive, and whether it entailed inhibition at the level of the TNF gene, the LPS signal transduction pathway, or both.

Materials and Methods

Cell Lines and Somatic Cell Hybrids. RAW 264.7 and NIH 3T3 cells were originally obtained from American Type Culture Collection (Rockville, MD), and passaged in DMEM supplemented with 5% FCS and 4% penicillin/streptomycin solution (Gibco Laboratories, Grand Island, NY). Each of the cell lines was stably cotransfected with pSV2*neo* and the CAT_{TNF} reporter gene, a minigene construct in which transcription is driven by a full-length TNF promoter, and in which the CAT coding sequence is flanked by both the TNF untranslated regions (16). Each of the lines was also rendered puromycin resistant. In the case of RAW 264.7 cells, this was accomplished by growing the cells in increasing concentrations of puromycin, beginning with a concentration of 1 μg/ml, and ultimately reaching a concentration of 50 μg/ml, which is lethal to unacclimated NIH 3T3 cells. In the case of NIH 3T3 cells, resistance was achieved by transfecting the cells with pSV2*puro* (18), and selecting for growth in the presence of puromycin at a concentration of 10 μg/ml, which resulted in the death of nontransfected cells. NIH 3T3 cells were also transfected with pSV2*neo* alone.

A somatic cell hybrid, in which the CAT_{TNF} construct was contributed by the RAW 264.7 cell genome, was produced by mixing the cells at a density of 10^6 each in six-well plates (diameter, 3 cm). After attachment, cells were washed five times in serum-free DMEM, and were then overlayed with polyethylene glycol (PEG),[1] 1500 mol wt (American Type Culture Collection), melted, and diluted 1:1 with DMEM. The cells were incubated with PEG for 2 min at 37°C. They were then washed three times with serum-free DMEM, and then incubated for 24 h in DMEM supplemented with 5% FCS. Selection was then initiated by growing the cells in the presence of both G418 (1 mg/ml) and puromycin (10 μg/ml) Several control cultures were initiated in parallel with the experimental culture just described. These included cultures in which parental cells were plated in isolation from one another and treated with PEG as described above, and cultures in which isolated parental cells, or mixtures of the two cell types, were plated but not exposed to PEG. All of the control cultures died in the process of selection. However, after 15 d, numerous colonies were apparent in the experimental fusion cultures.

The same procedure was followed to create hybrid cells that lacked the CAT_{TNF} reporter; however, the parental lines consisted of RAW 264.7 cells that had been acclimated to growth in the presence of puromycin, and 3T3 cells that had been transfected with pSV2*neo*.

Cytogenetic analysis of the hybrid cell lines was carried out in the Department of Pathology Cytogenetics Laboratory, University of Texas Southwestern Medical Center. 10 of 20 cells analyzed had between 98 and 101 chromosomes; the modal number was 100 (4 of 20 cells analyzed). Therefore, hybrids were considered to be pentaploid (as expected, given their derivation from triploid [RAW 264.7] and diploid [NIH 3T3] parental lines).

[1] *Abbreviations used in this paper:* LAR, locus activation region; PEG, polyethylene glycol; TSE, tissue-specific extinguisher; UTR, untranslated region.

Transfection. Permanently transfected RAW 264.7 cells and NIH 3T3 cells used for all studies were produced previously (16). Transient transfection of NIH 3T3 cells and RAW 264.7 × NIH 3T3 hybrid cells was carried out using calcium phosphate precipitates of cesium chloride–purified DNA as detailed elsewhere (19).

TNF Assay. The L929 cytotoxicity assay was used to measure mouse TNF production by the cell lines used in these studies (20).

CAT Assay. The TLC procedure of Gorman (20a) was used to assay CAT activity. In all cases, 10^6 permanently transfected cells were lysed by freezing in 120 μl of 0.25 M tris, pH 7.4, and after centrifugation, one-half of the supernatant was used for CAT assay. In transient transfection studies, 10^6 cells were initially transfected, and the entire culture was treated with LPS or left untreated after 48 h. Cells were lysed for CAT assay 64 h after initiation of transfection.

RNA (Northern) Blot Analysis. RNA was prepared as previously described (15). Samples were carefully quantitated both by measurement of OD at 260 nm, and by ethidium bromide staining after electrophoresis in agarose gels. Samples were diluted to contain precisely 15 μg of RNA per lane, and denatured in 100% formamide. 1 M glyoxal and bromophenol blue was added, and samples were heated to a temperature of 65°C for 5 min. They were then subjected to electrophoresis in 1.2% agarose, dissolved in Tris/acetate/EDTA buffer (21). Transfer to a nylon filter was accomplished electrophoretically, and the RNA was crosslinked by ultraviolet irradiation using a Stratalinker apparatus (Stratagene, La Jolla, CA). The membrane was briefly immersed in a buffer containing 50% formamide, 1% SDS, and 5× SSC at 68°C. An antisense riboprobe hybridizing with CAT (0.8 kb) or TNF (1.1 kb) mRNA was added to identify the respective mRNA species (21). After overnight incubation at 68°C, the membrane was washed twice with 2× SSC containing 1.5% SDS at 72°C for 20 min per wash, and twice with 0.2× SSC containing 1% SDS, at the same temperature, and for the same period of time. Total RNA was visualized afterward by staining the membrane with methylene blue (21).

Genomic DNA (Southern) Blot Analysis. Genomic DNA was prepared as described elsewhere (21). Digestion with restriction endonucleases that were either sensitive to CG methylation or methylation insensitive was carried out under conditions appropriate for each enzyme. DraI was chosen as a methylation-insensitive enzyme, and MspI and HpaII were chosen as enzymes that recognize the same sequence motif (CCGG), but that have different sensitivity to methylation (only HpaII is inhibited by methylation). 30 μg (when probing the TNF gene) or 10 μg (when probing the CAT reporter gene) of genomic DNA was applied to each lane of an agarose gel and subjected to electrophoresis for a period of 14 h, using a field strength of 2 V/cm. After ethidium bromide staining, UV nicking, denaturation, and capillary transfer, prehybridization and hybridization were carried out as elsewhere described (21).

Other Materials. Enzymes for restriction analysis were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). G418 was obtained from Gibco Laboratories (Grand Island, NY). Puromycin and cycloheximide were obtained from Sigma Chemical Co. (St. Louis, MO). LPS (Escherichia coli strain 0.127:B8) was obtained from Difco Laboratories (Detroit, MI). LPS and cycloheximide were applied to cells at concentrations and for time intervals stated in the figure legends.

Results

The TNF Gene Is Highly Methylated and Genetically Inactive in 3T3 Cells. To assess the degree of methylation of the

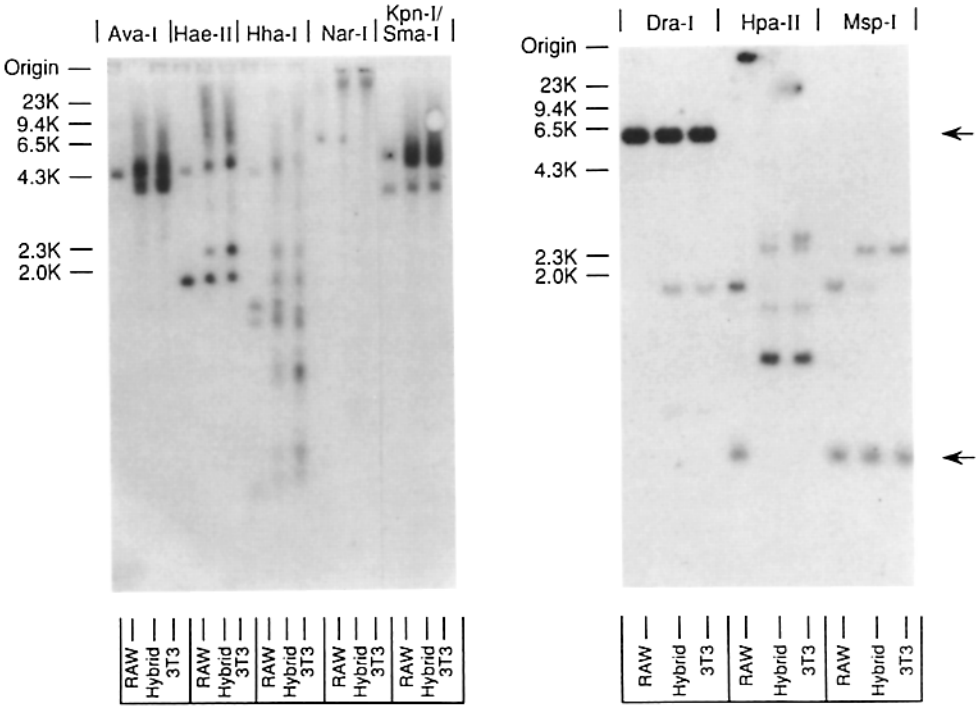

Figure 1. Differential methylation of the TNF gene in 3T3 cells, RAW 264.7 cells, and somatic cell hybrids. Cleavage, electrophoresis, and blot hybridization of DNA from each cell type was carried out as described in Materials and Methods. Membrane was exposed to film without intensifying screens for 48 h. (*Left*) Cleavage with methylation-sensitive enzymes. (*Right*) Cleavage with the methylation-insensitive enzyme DraI and with the differentially methylation-sensitive enzyme pair HpaII and MspI.

TNF gene within 3T3 cells and within RAW 264.7 macrophages, genomic DNA was obtained from cells of each type and digested to completion with a panel of methylation-sensitive and -insensitive restriction endonucleases (Fig. 1, lanes *1* and *3* for each enzyme). Obvious differences in the cleavage pattern were observed with each of the methylation-sensitive enzymes used. In general, the TNF gene was less readily cleaved in 3T3 cells than in RAW 264.7 macrophages (although some exceptions to this trend were observed), and overall, the density of methylated restriction sites appeared higher in the former cell type. Very similar cleavage patterns were observed when 3T3 and RAW 264.7 cell DNA samples were restricted with DraI (*top arrow*), and with MspI (*bottom arrow*). The former enzyme recognizes a nonmethylatable target sequence, whereas the latter is not blocked by methylation of its target sequence. RAW 264.7 cell DNA was identically cleaved by MspI and by HpaII, suggesting that no modification occurs at the sites recognized by this enzyme pair. On the other hand, a marked difference in the cleavage pattern was observed when 3T3 cell DNA was cut with HpaII, as opposed to MspI. This suggests modification of the CCGG motifs that occur in the 3T3 cell TNF gene.

Not surprisingly, the TNF gene could not be induced by LPS within 3T3 cells, nor by cycloheximide treatment. This latter finding, taken together with evidence that the TNF gene is heavily methylated in 3T3 cells, indicates that the gene has been inactivated, i.e., is not accessible to the transcriptional apparatus. On the other hand, both LPS and cycloheximide readily induced TNF gene expression in RAW 264.7 cells (Fig. 2).

A CAT Reporter Gene Bearing TNF Regulatory Elements Is Expressed in Response to Cycloheximide in Both 3T3 and RAW 264.7 Cells; LPS Elicits a Response in RAW 264.7 Cells Only. To test the integrity of the LPS signaling pathway in each cell type, a CAT reporter gene, transcriptionally driven by the TNF promoter and containing the TNF 5′-untranslated region (UTR) and 3′-UTR, was introduced into both of the lines by cotransfection with pSV2*neo*, and stable transfectants were isolated by G418 selection (16). The accessibility of the reporter construct was assessed in each cell line by treatment with cycloheximide. As shown in Fig. 3, lanes *C*, the CAT mRNA was readily induced by this procedure, both in RAW 264.7 cells and in 3T3 cells. It should be noted that the reporter gene copy number is far higher in the RAW 264.7 cell genome than in the 3T3 cell genome (not shown), hence the disparity between cycloheximide induction in the two cell lines.

On the other hand, while the reporter is clearly capable of responding to this type of activating stimulus, CAT mRNA is not produced within transfected 3T3 cells in response to LPS. Only RAW 264.7 cells respond (Fig. 3, lanes *L*), indicating that in 3T3 cells, some aspect of the LPS signaling apparatus is absent or defective.

Hybrids Produced by Fusing 3T3 and RAW 264.7 Cells Fail to Express the TNF Gene when Exposed to LPS. Since 3T3 cells maintain their TNF genes in an inactive state, they may be assumed to have failed to activate the locus, or to have silenced it at some point in transit through development or immortalization. We reasoned that if a specific mechanism for TNF gene inactivation exists in 3T3 cells in an active

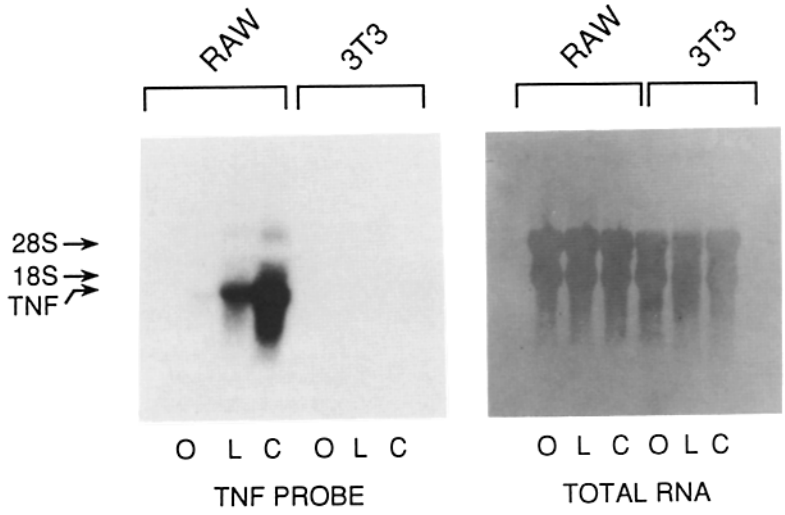

Figure 2. RNA (Northern) blot analysis of the TNF message in RAW 264.7 and 3T3 cells. Cells were either not induced (*O*), or induced for 2 h using 1 μg/ml LPS (*L*), or 100 μg/ml cycloheximide (*C*).

form, it might be detected through cell hybridization experiments. Accordingly, G418-resistant 3T3 cells were fused to puromycin-resistant RAW 264.7 cells using polyethyleneglycol, and the culture was selected for growth in the presence of both agents.

When the hybrid cells were exposed to LPS, no TNF was produced (data not shown), suggesting that the TNF genes contributed by the RAW 264.7 cell parent were no longer accessible, that the LPS signaling pathway was inoperable, or both. LPS could not induce accumulation of the TNF mRNA within hybrid cells, and cycloheximide could induce the gene at only 2% the level seen in the RAW 264.7 cell parent (Fig. 4). Methylation of the TNF genes derived from

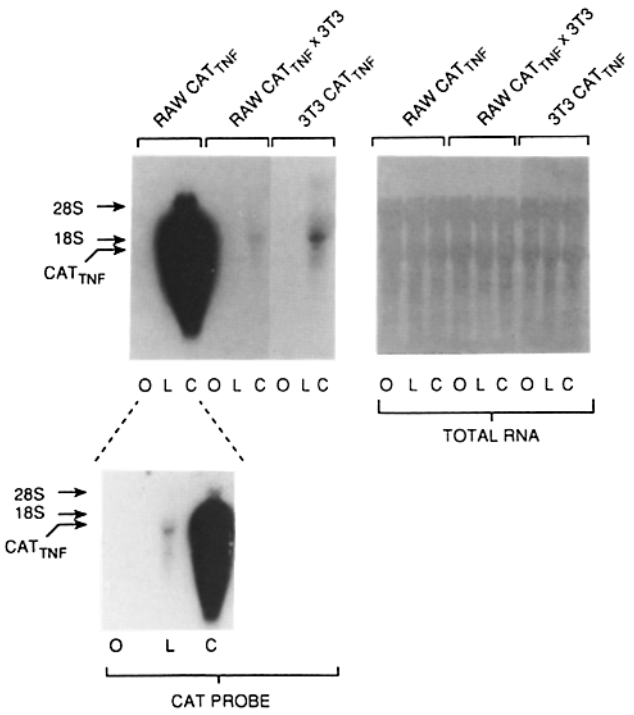

Figure 3. Induction of the CAT_{TNF} reporter mRNA in RAW 264.7 cells, 3T3 cells, and a somatic cell hybrid. Treatment of the cells with no inducer, with LPS, or with cycloheximide is indicated as in Fig. 2. An expanded version of the first three lanes is shown for clarity (the same RNA samples were rerun on a separate gel with wider spacing).

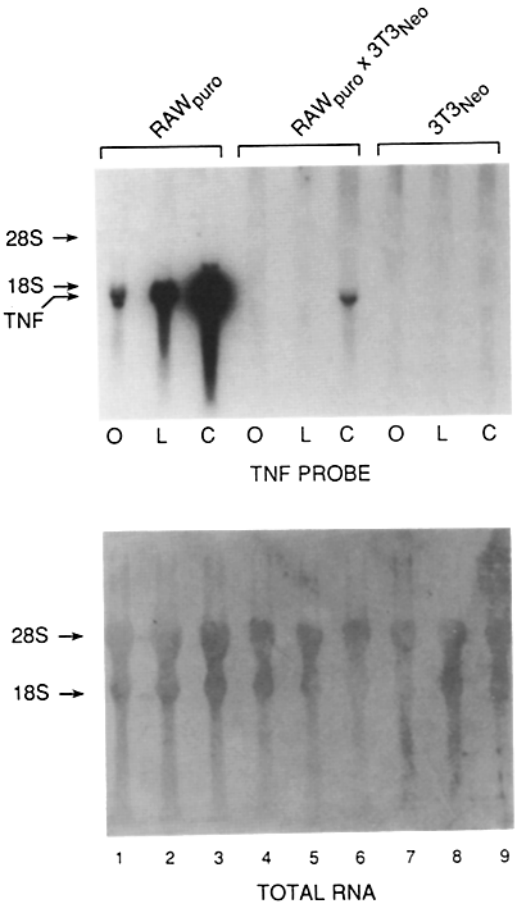

Figure 4. Accessibility and LPS responsiveness of the TNF gene in RAW 264.7 cells, 3T3 cells, and somatic cell hybrids. RNA (Northern) blot detection of the TNF message in these cell types was carried out using cells that had been left unstimulated (*O*), that were stimulated with LPS (*L*), or that were stimulated with cycloheximide (*C*).

the hybrid cells was extremely similar to that observed in the 3T3 parental line, rather than to that of the RAW 264.7 line (Fig. 1, lanes *2* and *3* for each enzyme). Only the NarI site (cleavable in RAW 264.7 DNA, but uncleavable in 3T3 DNA) was retained in a partially cleavable state in DNA derived from hybrid cells. Therefore, it would seem that factors contributed by the 3T3 cell are capable of directing specific inactivation of the TNF locus. Moreover, 3T3 cells enforce the adoption of their own methylation pattern within the heterokaryon; the RAW 264.7 cell pattern is largely discarded.

The CAT Reporter Gene Is Minimally Accessible when Transferred to a Heterokaryon through Cell Fusion, Adopts a New Methylation Pattern, and Does Not Respond to LPS. Since cycloheximide could not induce normal levels of TNF gene expression in hybrid cells, it was assumed that the gene had, for the most part, been rendered inaccessible. To determine whether sequences within the TNF gene were recognized in this process, the CAT reporter was introduced into the hybrid cell milieu with the RAW 264.7 genome, whereupon its accessibility could be examined by cycloheximide induction. Cycloheximide inducibility was drastically reduced (~1,000-fold) with respect to the level of induction observed in the parental line itself (Fig. 3).

Since the reporter gene originating within RAW 264.7 cells was presumably unaltered by cell fusion, either with respect to copy number or position in the genome, it must be inferred that factors contributed by 3T3 cells participate to radically suppress the accessibility of the reporter gene in the hybrid cell environment. Indeed, at some point in their history, 3T3 cells clearly suppressed accessibility of the authentic TNF locus, and have maintained the gene in an inactive state ever since (Figs. 2 and 4).

DNA from RAW 264.7 cells and from hybrid cells in which the reporter was contributed by the same RAW 264.7 cell parent was then reanalyzed to examine the methylation pattern of the reporter gene in each environment. As illustrated in Fig. 5, cell fusion forces the adoption of a new methylation pattern by a reporter gene resident within the RAW 264.7 cell genome. All of the enzymes used yielded different patterns when applied to the two DNA samples, except the methylation-insensitive enzymes DraI and MspI (which cut DNA derived from each cell type in an identical fashion). Notably, HpaII (which recognizes the same sequence motif as MspI, but which is methylation sensitive) cuts the RAW 264.7 cell reporter far differently than it cuts the same gene obtained from hybrid cells.

We then sought to assess the integrity of the LPS signaling pathway in hybrid cells. When the reporter was introduced into hybrid cells by fusion, neither the reporter mRNA (Fig. 3) nor the reporter protein (Fig. 6, *top*) were inducible by LPS. Transient transfection studies (Fig. 6, *bottom*) were also carried out, since we reasoned that inactivation of the reporter might be time dependent, and might require integration or completion of the cell cycle. The 3′-UTR of the TNF gene is known to diminish expression of reporter constructs to which it is attached (3, 23); and therefore, when transient transfection studies were performed, a reporter construct

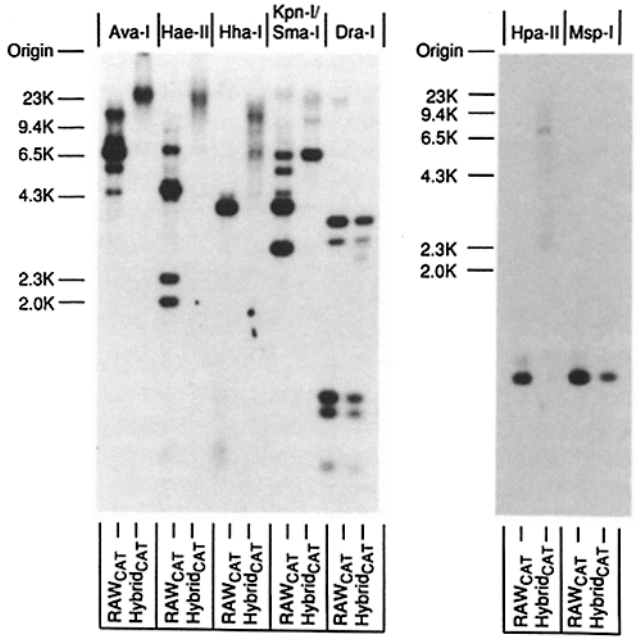

Figure 5. Modification of the CAT reporter construct resident in the RAW 264.7 cell genome after fusion with 3T3 cells. Enzymes used for cleavage of DNA, and cell line of origin, are indicated. A probe for CAT coding sequence was used in hybridization instead of a TNF probe (used in Fig. 1). (*Left*) Methylation-sensitive enzymes and the methylation-insensitive enzyme DraI. (*Right*) Differentially sensitive HpaII/MspI enzyme pair.

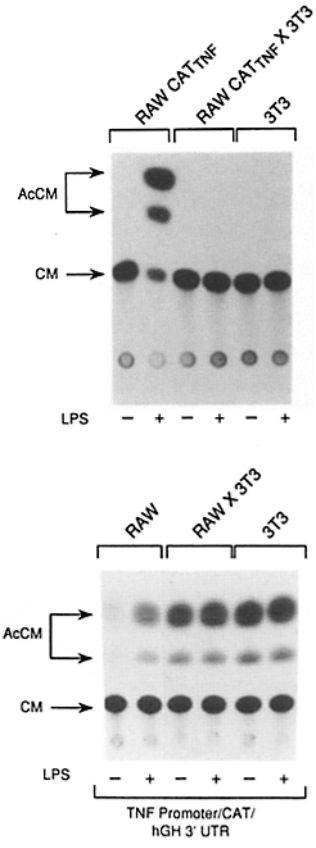

Figure 6. LPS signaling in RAW 264.7 cells and a somatic cell hybrid. (*Top*) The CAT_{TNF} reporter construct was introduced into RAW 264.7 cells as indicated, and judged to be accessible as shown in Fig. 3. The parental cells and their fusion product were then stimulated with no inducer (−), or with LPS for 16 h at a concentration of 1 μg/ml (+). Cells were then lysed, and CAT activity was measured as described in Materials and Methods. (*Bottom*) Transient transfection of RAW 264.7, hybrid, and 3T3 cells with a TNF promoter–CAT construct. Cells were stimulated with LPS for 16 h at a concentration of 5 μg/ml (+), or left untreated (−) before lysis and assay of CAT activity.

lacking the TNF 3′-UTR (designated TNF promoter/CAT/hGH 3′ UTR) was used instead of the CAT_{TNF} reporter. This construct was induced 50-fold in pools of permanently transfected RAW 264.7 cell clones (24), and was strongly LPS responsive in transiently transfected RAW 264.7 cells as well (Fig. 6, *bottom*). However, though highly expressed, the construct was entirely noninducible by LPS in the hybrid cell line, as in 3T3 cells, effectively proving that these cell types lack an intact LPS signaling pathway.

Discussion

The TNF promoter is ubiquitously used when introduced into cells by transfection (3). As dictated by cell differentiation, however, only a limited subset of cells express the TNF gene. Two general mechanisms seem to assure suppression of TNF biosynthesis in cells that do not secrete the hormone. One of these is translational suppression, which is chiefly dependent upon sequences present within the TNF 3′-UTR (3, 15, 22, 23, 25, 26). The importance of this mechanism in vivo has been amply demonstrated by studies in which mutation of the TNF 3′-UTR was shown, in transgenic mice, to eventuate an autoimmune arthritis (27). The other mechanism entails inactivation of the TNF gene, so that the promoter is no longer capable of driving transcription.

The TNF gene is expressed at a far higher level in macrophages (14) than in cardiac myocytes (28), astrocytes (6), keratinocytes (7), or any of the other cell types in which TNF production has been reported. It may be presumed that, in these other cell types, the gene is not fully accessible as it is in macrophages. In certain cells, as in the 3T3 cells of the present study, the gene has been silenced altogether.

The molecular basis of mammalian gene activation (or inactivation) is poorly understood in most instances. It has perhaps been best studied in the case of the β-globin locus. In this example, it is clear that far-upstream sequences comprising a dominant control region, or locus activation region (LAR), respond to erythroid-specific factors to permit activation of the β-globin cluster (29–31). A similar LAR may act to permit expression of the TNF gene, and may be most fully utilized in macrophages. The location of a LAR, if such exists, is not known. In the present study, we focus upon the existence of elements that suppress (rather than activate) expression of the TNF locus.

The RAW 264.7 cell TNF genes are permanently silenced after fusion with a 3T3 cell (which itself maintains its TNF genes in an inactive form). The RAW 264.7 cell TNF locus also adopts a pattern of methylation similar to that of the 3T3 cell TNF locus when these two cell types are fused. Methylation and inactivation of a CAT reporter construct bearing TNF promoter, 5′-UTR, and 3′-UTR sequences likewise occurs when this artificial gene is transferred from the RAW 264.7 cell to a hybrid cell environment through fusion with a 3T3 cell. Of note, however, the reporter construct remains accessible when directly transfected into 3T3 cells. It may therefore be concluded that cell fusion induces or reconstitutes a specific system for recognition of the macrophage TNF locus, leading to its extinction. While it might have been anticipated that such a system would operate only during cell differentiation, or in the process of immortalization, cell fusion is evidently capable of activating it.

The exact sequences recognized by the suppressive system expressed within the hybrid cell environment remain to be determined. In principle, they might reside anywhere within the TNF gene, or at locations remote from the gene. However, it would appear that the TNF coding sequence and introns are not critical for recognition, nor are remote sequences, since the reporter construct is, like the TNF gene itself, largely inactivated after cell fusion. The sequences recognized in the inactivation process have thus been circumscribed to a region of DNA that encompasses the distal coding sequence and 3′-UTR of the lymphotoxin gene, the TNF promoter/enhancer region, the TNF 5′-UTR, and the TNF 3′-UTR (a total of 3.2 kb of DNA).

Presumably, 3T3 cells also silence the expression of other genes required for a response to LPS (i.e., genes encoding proteins that transmit the LPS signal). As such, the response to LPS is abolished when macrophages are fused with 3T3 cells. Moreover, while a CAT reporter gene remains transcribable in the hybrid cell environment after introduction by transient transfection, it is not induced after challenge with LPS.

Extinction of differentiated cell functions has been studied in hepatocellular carcinoma cells, wherein fusion with a fibroblast cell line leads to a shutoff of tyrosine aminotransferase gene expression (32). Tissue-specific extinguishers (TSE) have, in this case, been linked to human chromosome 17 (TSE1) (33) and to mouse chromosome 1 (TSE2) (34) by cytogenetic analyses, and the TSE 1 product is thought to be identical to the RIα subunit of protein kinase A (35).

It is well known that high levels of cAMP block LPS responses in macrophages. Therefore, dibutyryl cAMP (36), pentoxifylline (36), amrinone (37), and other phosphodiesterase inhibitors effectively block LPS-induced TNF biosynthesis, acting at a transcriptional level (38). However, the effect of TSE1 gene overexpression would be to diminish the sensitivity of the protein kinase A pathway to increases in cAMP concentration. As such, it would seem improbable that the TSE1 locus contributes to suppression of the LPS response or, for that matter, to TNF gene extinction. Preliminary studies, not reported with the data shown, tend to support this view, insofar as transfection of RAW 264.7 and hybrid cells using RIα mutant expression constructs (39, 40) or protein kinase A inhibitor expression constructs (41) has no influence upon LPS-induced TNF gene expression. Moreover, in unstimulated hybrid cells, cAMP concentration differs little from that observed in RAW 264.7 cells (data not shown).

Conceivably, the same developmental mechanism that assures silence of the TNF gene may direct inactivation of other genes required for the response to LPS. As such, they would exist as constituents of a common developmental unit. However, such a relationship has yet to be established.

Further studies may ultimately elucidate the target sequences required for developmental inactivation of the TNF locus. It may be anticipated that disruption of elements required

for proper tissue targeting of TNF gene expression might lead to aberrant development, or to diseases with inflammatory characteristics, particularly if combined with mutations of the TNF 3′-UTR.

We are grateful to Drs. Stan McKnight and to Richard Maurer for their help in providing the RIα and PKI constructs, respectively. We are also indebted to Dr. Jeff Wiseman, who kindly agreed to assay cAMP levels in NIH 3T3 cells, RAW 264.7 cells, and the hybrid cells used in these studies.

This work was funded, in part, by a grant from the American Cancer Society (CD-510), and by a National Institutes of Health grant (5-P01-DK-42582).

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Received for publication 30 September 1992 and in revised form 28 January 1993.

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