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Constitutive activity of the tumor necrosis factor promoter is canceled by the 3′ untranslated region in nonmacrophage cell lines; a trans-dominant factor overcomes this suppressive effect

(cytokine/transcription/posttranscriptional regulation/transactivator)

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ABSTRACT **The role of the mouse tumor necrosis factor (TNF) promoter, 5′ untranslated region (UTR), and 3′ UTR in TNF gene expression has been examined in three nonmacrophage cell lines (HeLa, NIH 3T3, and L-929). The TNF promoter is not macrophage-specific. On the contrary, it constitutively drives reporter gene expression in all three cell lines. Not only the full-length promoter but also truncated versions of the promoter, lacking NF-κB binding motifs, are active in each type of cell. The TNF 3′ UTR effectively cancels reporter gene expression in HeLa cells and in NIH 3T3 cells but fails to block expression in L-929 cells. L-929 cells contain a factor that overcomes the inhibitory influence of the TNF 3′ UTR. Its action depends upon the presence of sequences found in the TNF 5′ UTR. Cell-fusion experiments reveal that this activator is trans-dominant. These studies highlight the essential role played by the TNF 3′ UTR, which silences the TNF gene in cells that might otherwise express TNF. They also reveal the existence of an escape mechanism whereby inappropriate synthesis of TNF might occur.**

Although tumor necrosis factor (TNF) is predominantly a product of macrophages, it is also known to be expressed by nonmacrophages (1–4) and even by extrahematopoietic cells (5, 6). Such cells may constitute an important source of TNF, as this protein is produced in neoplastic disease. However, the genetic sequences required for expression in such cell types have not been determined and the regulatory mechanisms have not been explored. To study TNF gene regulation in diverse cell types, a reporter construct was designed in which a chloramphenicol acetyltransferase (CAT) coding sequence was substituted for the authentic TNF coding sequence and the TNF promoter, 5′-untranslated region (UTR), and 3′-UTR sequences were preserved (7).

In the course of our work, it became apparent that a reporter construct bearing all of these elements (promoter, 5′ UTR, and 3′ UTR) was strongly and constitutively expressed when permanently transfected into cells of the L-929 fibrosarcoma line (7). This observation suggested that, at least in certain extrahematopoietic cell types, all of the requisite machinery for TNF gene expression must be in place; however, the gene itself is normally not expressed in L-929 cells, presumably as a result of developmental inactivation (7). In other nonhematopoietic cells examined (HeLa and NIH 3T3 cells), the reporter construct was silent, suggesting either that factors required for TNF gene expression are lacking from these cells or that a repressive system is operative. To determine which portion of the TNF gene governs expression, we designed other CAT reporter constructs containing various combinations of the TNF promoter and 5′ and 3′ UTRs and analyzed their expression in each cell type.

We also investigated the existence of activating or suppressing factors involved in the regulation of TNF biosynthesis through cell fusion experiments, in which cells that constitutively express the reporter gene were hybridized with cells that do not express the construct. If a dominant activator of TNF biosynthesis was present in a constitutively expressing cell type, fusion with a nonexpressing cell would produce a hybrid cell in which the reporter gene (supplied by either of the parental cell types) would be expressed. On the other hand, if a dominant suppressor of TNF biosynthesis was present in the nonexpressing cell type, the hybrid cell would not express the reporter.

MATERIALS AND METHODS

Constructs. The constructs used in these studies are schematically represented in Fig. 1. These constructs were designed to include either the major portion of the mouse TNF promoter (containing all of the NF-κB elements important for gene induction in macrophages) (8, 9) or a markedly truncated version of the promoter (containing only an SP-1 element and a "TATA" box). Since they were prepared at different times and in different laboratories, minor discrepancies in promoter length (1059 bp, 1009 bp, or 939 bp for the long form and 109 bp or 100 bp for the short form) are apparent in Fig. 1. As may be judged from the data, these minor discrepancies are inconsequential, since the length of the promoter proved to have little impact upon its action in extrahematopoietic cell types.

The full-length reporter gene (consisting of a CAT coding sequence flanked by the TNF promoter, 5′ UTR, and 3′ UTR) has been described elsewhere in detail (7), designated "CAT_{TNF}," and its sequence is available upon request. This construct is designated Pro-5′-CAT-3′. A shortened version of this reporter gene, containing 1009 bp of promoter sequence, was derived by cleavage with *Kpn* I and *Bam*HI and religation to yield the truncated molecule. CAT reporter constructs, containing 1059 bp and 109 bp of the TNF promoter and the TNF 5′ UTR but not the TNF 3′ UTR, were as described (10). Reporter constructs containing a CAT coding sequence flanked at the 5′ end with 939 bp or with 100 bp of the TNF promoter and at the 3′ end with the TNF 3′ UTR, but lacking the TNF 5′ UTR, were derived from Pro-5′-CAT-3′. The original construct was digested with *Kpn*

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Abbreviations: TNF, tumor necrosis factor; UTR, untranslated region; CAT, chloramphenicol acetyltransferase.

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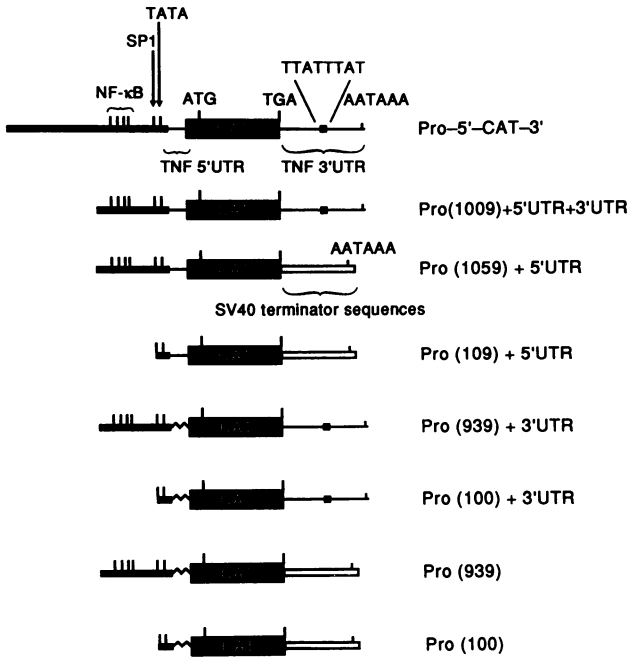

FIG. 1. CAT reporter constructs used in these studies. The TNF promoter, 5′ UTR, and 3′ UTR were assembled as indicated. When the TNF 3′ UTR was omitted, a simian virus 40 (SV40) terminator sequence (open box) was substituted. When the TNF 5′ UTR was omitted, no sequence was substituted (deletion indicated by a wavy line), but the 76-base-pair (bp) CAT 5′ UTR remained present in all constructs. The lengths of upstream promoter sequence attached are indicated to the right.

I and *Cla* I to remove the TNF promoter and 5′ UTR. It was then ligated with fragments of 939 bp and 100 bp of the TNF promoter, which were synthesized by the polymerase chain reaction with linkers generating a *Kpn* I site at the 5′ end and a *Cla* I site at the 3′ end. The TNF promoter region in each of the constructs was sequenced by the dideoxynucleotide elongation method (11). Reporter constructs containing 939 bp and 100 bp of the TNF promoter, but no 5′ UTR or 3′ UTR sequences, were synthesized by digesting the two previously described vectors with *Hin*dIII and *Sma* I to remove the CAT sequence and the TNF 3′ UTR. This fragment was replaced by the *Hin*dIII–*Sma* I fragment derived from pBLCAT3 (12), which contains the CAT coding sequence followed by simian virus 40 polyadenylylation signals. The plasmid pSVLCAT (used for monitoring transfection efficiency) was purchased from Pharmacia.

Transfections. The Pro-5′-CAT-3′ construct was permanently transfected into HeLa cells, NIH 3T3 cells, and L-929 cells. Cell lines were characterized with respect to reporter gene copy number (7). Nontransfected cells of the same types were also maintained in the laboratory. In addition, a HeLa cell line rendered resistant to methotrexate at 150 ng/ml by transfection with the vector pSVR400 (obtained from C. Simonson, Invitron Corp., Redwood City, CA) was produced in our laboratory and utilized in one experiment.

Transient transfections were performed according to the calcium phosphate method of Chen and Okayama (13). For each trial, 20 μg of DNA was used to transfect 2×10^6 cells, and half of the cells on each transfected plate were used for the CAT assay.

Cell Hybridization. When the parental cell containing the reporter gene was incapable of expressing CAT activity (e.g., when HeLa cells or NIH 3T3 cells carrying the Pro-5′-CAT-3′ construct were used as a partner in fusion), transient cell fusion experiments were sufficient to reveal whether activation would occur after hybridization with a second cell type. If, on the other hand, the parental cell containing the reporter gene expressed CAT constitutively (e.g., when L-929 cells carrying the Pro-5′-CAT-3′ construct were used as a partner in fusion), a period of selection was required to remove the unfused parental cells from the culture.

Transient cell fusions were performed by plating 1×10^6 cells of each parental type in 3-cm six-well tissue culture dishes. After cells attached to the dish in close contact with one another, fusion was induced by overlaying with 50% (wt/vol) autoclaved polyethylene glycol (M_r 1500 PEG; obtained from Fluka or from the American Type Culture Collection) dissolved in Hanks' balanced salt solution supplemented with 40 mM $CaCl_2$, 1.05 mM $MgCl_2$, 0.83 mM $MgSO_4$, and 4.2 mM $NaHCO_3$. PEG was allowed to remain in contact with the cells for 5 min at 37°C. Thereafter, it was gradually diluted with fresh medium and finally aspirated. The fused monolayers were washed twice more with fresh medium. Invariably, substantial numbers of binucleated cells were observed after this treatment. Control monolayers of each of the two parental lines were prepared in parallel. In additional control wells, parental cells were cocultured or cultured separately from one another but treated with ordinary culture medium instead of PEG solution. Cells were either harvested by trypsinization 16 h after fusion or initiation of coculture, were sedimented by centrifugation, suspended in 0.12 ml of 0.25 M Tris·HCl (pH 7.8), and frozen. After thawing, the lysate was centrifuged at 16,000 × *g* for 2 min. Half of the supernatant was used for CAT assays.

Permanent cell hybridization was sometimes performed to establish a pure population of fused cells, involving HeLa as one participant. In these experiments, the HeLa cells had previously been transfected with the vector pSVR400, conferring methotrexate resistance, whereas the other cell types had been cotransfected with pSV2neo and were therefore resistant to G418. Each of the parental lines and a mixture of the two lines were exposed to PEG in each of three separate wells. After a 24-h recovery period, the cells were exposed to a combination of methotrexate (0.15 μg/ml) and G418 (1.0 mg/ml) in medium that contained dialyzed fetal bovine serum. After 3 weeks, when all of the cells present in each parental monolayer had died or appeared nonviable, cells were harvested from all three monolayers and from control (nonselected parental) cultures by trypsinization. The fused-cell population was counted, and CAT assays were performed using lysate prepared from known numbers of cells.

CAT Assay. CAT activity was determined by the method of Gorman *et al.* (14).

RESULTS

Analysis of the Regulatory Functions of the TNF Promoter Region and UTRs in L-929, HeLa, and NIH 3T3 Cell Lines. The Pro-5′-CAT-3′ construct is constitutively expressed in L-929 cells but is not expressed in NIH 3T3 cells and in HeLa cells (7). To determine which parts of the construct are involved in the constitutive expression in L-929 cells or in preventing expression in HeLa and in NIH 3T3 cells, we designed other CAT reporter constructs containing various combinations of the TNF promoter and the TNF 5′ and 3′ UTRs (see Fig. 1). Their expression was assayed by transient transfections.

As shown in Fig. 2, the promoter can be utilized by all three cell lines. Furthermore, the 100 bp most proximal to the cap site are sufficient to drive CAT gene expression. The TNF 3′ UTR strongly suppresses CAT expression in each cell type. In L-929 cells, however, the suppressive effect of the 3′ UTR is apparently overcome, leading to the expression of the construct. The suppressive effect of the 3′ UTR may only be overcome if the 5′ UTR is also included in the construct. In

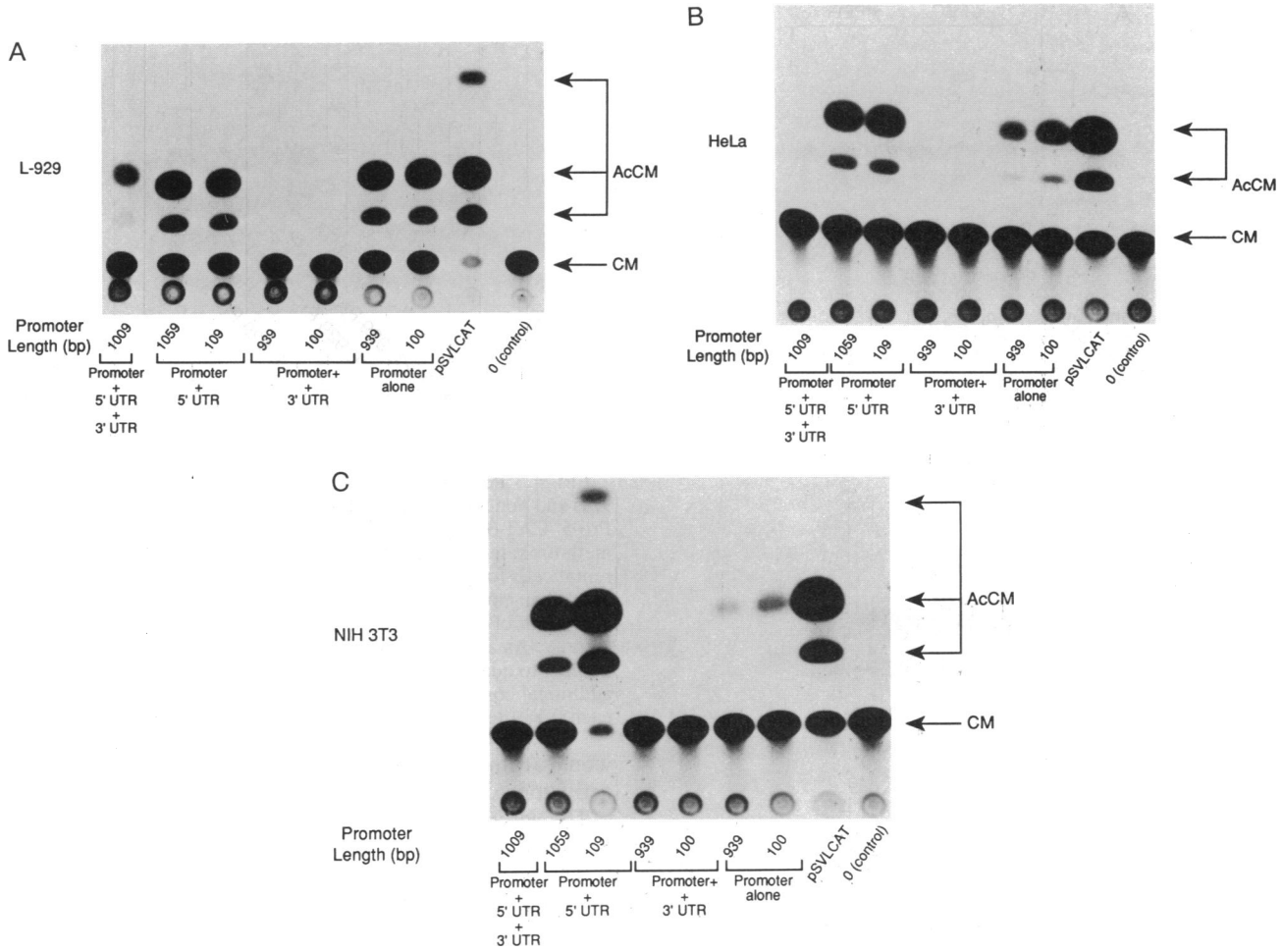

FIG. 2. Expression of CAT reporter constructs containing various combinations of the TNF promoter, 5′ UTR, and 3′ UTR in L-929 cells (*A*), HeLa cells (*B*), and NIH 3T3 cells (*C*). A pSVLCAT construct was transfected into each cell type to monitor transfection efficiency. The characteristics of the constructs are specified at the bottom. CM, chloramphenicol used as substrate in the CAT assay; AcCM, 1-acetyl, 3-acetyl, and 1,3-diacetyl forms of chloramphenicol. Formation of the acetylated products indicates expression of CAT.

HeLa cells and in NIH 3T3 cells, the effect of the 3′ UTR remains in force, whether or not the 5′ UTR is present.

The essential permissive effect of the 5′ UTR in L-929 cells was documented by permanent transfection studies, in which constructs containing the TNF promoter and TNF 3′ UTR but lacking (or not lacking) the TNF 5′ UTR were cotransfected into L-929 cells with pcDNAneo. In three sets of pooled clones, no expression was observed when the 5′ UTR was absent from the construct (data not shown).

Fusion of L-929 Cells to Either Nonexpressing Cell Type Leads to Constitutive Expression of the Included Reporter Construct. L-929 cells contain a dominantly acting factor that causes expression of the Pro-5′-CAT-3′ construct. This is apparent from the fact that when L-929 cells are fused with either HeLa or NIH 3T3 cells, the reporter gene being contributed by one parental type or the other, CAT activity is expressed. PEG treatment of the cells that contain the reporter construct is not, in itself, sufficient to induce CAT expression. Furthermore, coculture of these cells with L-929 cells fails to elicit CAT biosynthesis in the absence of PEG (Fig. 3). Thus, hybrid formation between the two cell types is required for constitutive expression of the reporter construct.

The efficiency of cell fusion was monitored by binucleated cell formation; although this gave a clear indication that cell fusion was occurring, the incidence of hybrid formation could not be determined, since no morphologic distinction could be made between the nuclei derived from each cell type. To analyze a pure population of hybrid cells, G418-resistant L-929 cells that contained the reporter construct (and expressed it constitutively) were fused with HeLa cells that had been rendered methotrexate resistant. After 3 weeks of selection in the presence of both methotrexate and G418, a nearly pure population of nonproliferating fused cells was obtained and tested for expression of the reporter construct. Some residual CAT activity was apparent in the selected population of L-929 cells, suggesting that ≈1% of the original population had survived treatment with G418 and methotrexate. Very high levels of CAT activity were demonstrated in the hybrid population, confirming the recessive nature of the nonexpressing phenotype (Fig. 4). Indeed, the same number of Pro-5′-CAT-3′-bearing L-929 cells (lysate derived from 2.5×10^4 cells added to the assay system) yielded only ≈10% of the CAT activity in the hybrid cells, suggesting that the HeLa cells contributed factors that, rather than suppressing, enhanced expression of the minigene construct.

DISCUSSION

We have investigated mechanisms by which three nonmacrophage cell lines govern expression of the TNF gene. Our findings demonstrate that the TNF promoter is not macrophage specific. On the contrary, the promoter (or indeed, a short fragment of the promoter) is well utilized by each cell type. Strikingly, the 3′ UTR of the TNF gene enforces cell-type specificity. Hence, its inclusion effectively cancels

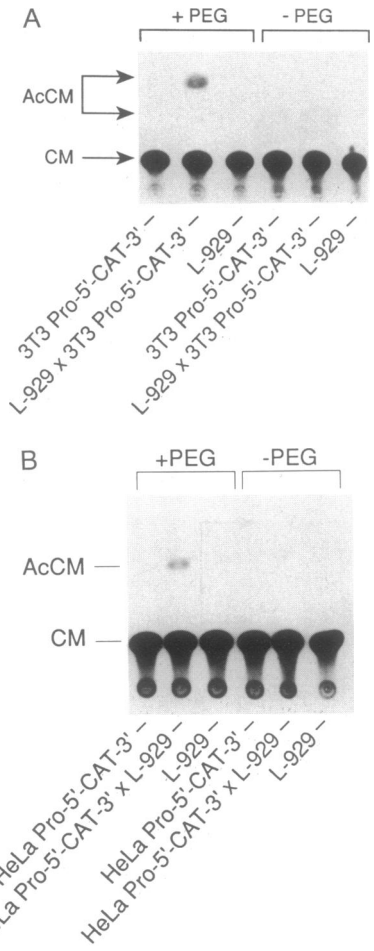

FIG. 3. Transient fusion between constitutively expressing (L-929) and nonexpressing (NIH 3T3 or HeLa) cell lines. Nontransfected L-929 cells were fused with NIH 3T3 cells (*A*) or HeLa cells (*B*) as indicated. Each of the latter cell types had been permanently transfected with the Pro-5′-CAT-3′ reporter construct. × designates fusion or coculture of two cell lines. + PEG, cells fused with polyethylene glycol; − PEG, cells cocultured but not fused. Abbreviations are as in Fig. 2.

reporter gene expression in two of the three cell lines studied (HeLa and NIH 3T3).

In the third cell line examined (L-929), the 3′ UTR fails to abolish expression of the reporter, provided that the 5′ UTR is also included in the construct. Cells escape from the suppressive effect of the 3′ UTR by the action of a trans-dominant factor that, when introduced into either of the nonexpressing cell types studied by somatic cell fusion, permits expression of the previously silent reporter gene.

The mechanism by which the 3′ UTR suppresses reporter gene expression in HeLa cells and in NIH 3T3 cells remains to be determined. On the one hand, it is possible that the TNF 3′ UTR functions, as in macrophages, to suppress translation of the reporter mRNA (15, 16). Alternatively, the TNF 3′ UTR may act to destabilize the reporter mRNA (17). These effects are known to be minimally dependent upon the presence of a conserved U+A-rich element within the 3′ UTR of the mRNA. Preliminary deletion studies (data not shown) suggest that this element is critical for the suppressive effect that is observed in HeLa and NIH 3T3 cells.

Yet another intriguing problem concerns the mechanism by which the trans-dominant factor expressed by L-929 cells overcomes the suppressive effect of the 3′ UTR. Given the fact that sequences present in the 5′ UTR are required to permit escape from the suppressive effect of the 3′ UTR, it would seem possible that a physical interaction may engage activator, 5′ UTR, and 3′ UTR structures.

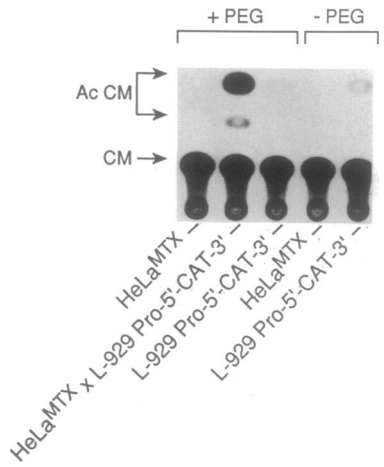

FIG. 4. Permanent fusion between constitutively expressing (L-929) and nonexpressing (HeLa) cell lines. L-929 cells containing the Pro-5′-CAT-3′ were fused with HeLa cells that had been rendered methotrexate-resistant ($HeLa^{MTX}$) and selected along side the parental cells for growth in the presence of both G418 and methotrexate. When most of the 1×10^6 parental cells originally plated had died as a result of selection, 2.5×10^4 fused cells were assayed for CAT activity. As a control, half of the residual parental cells (too few in number to quantitate precisely) were also used for CAT assay. As an additional control, 2.5×10^4 nonselected nonfused parental cells of each type (designated −PEG) were also assayed for CAT activity.

The activator that we have now defined might function in several ways. Rubin *et al.* (5) reported several years ago that L-929 cells rendered resistant to the cytolytic effect of lymphotoxin (TNF-β) would constitutively express TNF-α in low but detectable quantities. Although no conceptual framework for the explanation of this finding was available at the time, we now propose that the TNF gene is active in a small percentage of L-929 cells, where it is expressed as a result of the dominantly acting factor that we have observed. Such cells, having necessarily acquired TNF resistance, might find themselves at a selective advantage when grown in the presence of lymphotoxin.

In a similar vein, it is likely that certain cancers express TNF constitutively *in vivo* under the influence of the same activator (6, 18). The activator, as such, might have many biological actions apart from its ability to induce TNF synthesis, some of which might relate to maintenance of the transformed phenotype.

With regard to the production of TNF, it is conceivable that the L-929 cell activator may function to permit TNF biosynthesis as it occurs in the course of macrophage activation or activation of other cells known to be capable of producing TNF. In this scheme, *de novo* synthesis of the activator might allow TNF production to proceed. Finally, it is possible that the activator permits TNF gene expression in various tissues as mandated by a specific developmental program. It has been noted (B. P. Giroir, T. Brown, and B.B., unpublished work) that TNF is constitutively secreted by the normal neonatal thymus but by no other cells *in vivo*; it may be supposed that the suppression normally mediated by the 3′ UTR is mitigated under this circumstance, and the activator identified here might thus permit TNF biosynthesis to occur.

In the future, it would seem likely that the activator expressed by L-929 cells might be isolated through a molecular cloning approach, in which a selectable marker is substituted for CAT. Further studies of the expression of this factor in malignantly transformed cells and in normal macrophages might shed light on its function and effects.

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