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Immunology

Cell-free translation of a biologically active, antigen-specific suppressor T cell factor

(suppressor factor mRNA/T cell hybridoma/major histocompatibility complex)

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ABSTRACT ***In vitro*** **synthesis of an antigen-specific T cell suppressor factor (TsF) has been accomplished by using partially purified poly(A)-containing RNA in a rabbit reticulocyte lysate cell-free translation system. The poly(A)-containing mRNA was isolated from a cloned T cell hybridoma that constitutively produces a TsF specific for the synthetic polypeptide antigen poly(LGlu^{60}LAla^{30}LTyr^{10}) (GAT). The RNA was fractionated by size and translated *in vitro*. The 16S RNA fraction stimulated synthesis of a biologically active protein that specifically suppressed both the GAT-specific antibody response by spleen cells *in vitro* and the proliferation response to GAT by lymph node T cells from GAT-primed mice. Further, the suppressor factor had a binding site for GAT, a determinant encoded by the *I* subregion of the major histocompatibility complex (MHC), and an apparent M_r 19,000 estimated by functional assays on protein separated by NaDodSO$_4$/polyacrylamide gel electrophoresis. These results indicate that virtually no posttranslational modifications (other than proteolytic cleavage) are necessary to obtain biologically active TsF. Hence, the presence of carbohydrate or other chemical groups does not contribute to either the serological properties of GAT-TsF or its biological properties.**

Immune responses by inbred strains of mice to a wide variety of protein antigens are controlled by genes that lie within the major histocompatibility gene complex (MHC) (1). The use of relatively simple, synthetic polypeptide antigens has been particularly helpful in analyzing the function of these genes (2). Injection of inbred strains of mice with one of these antigens, poly(LGlu^{60}LAla^{30}LTyr^{10}) (GAT), divides the strains into two phenotypes—responders and nonresponders. The ability to develop an antibody response to GAT and to be primed for a subsequent T cell proliferative response is controlled by genes that map to the *K-I-A* subregions of the MHC (3, 4). When GAT is linked to an immunogenic carrier such as methylated bovine serum albumin (GAT-methylated albumin), both nonresponder and responder mice produce GAT-specific antibody (5) and are primed for a subsequent T cell proliferative response to GAT (6). Failure of nonresponder mice to respond to GAT is correlated with the preferential stimulation of antigen-specific suppressor T cells (7).

The mechanism(s) by which GAT-specific suppressor T cells regulate immunity has been investigated by analysis of T cell extracts from GAT-primed nonresponder mice (8–10). These extracts contain a specific factor, GAT-T cell suppressor factor (GAT-TsF), that inhibits plaque-forming cell (PFC) responses to GAT-methylated albumin *in vivo* and *in vitro* and also inhibits T cell proliferative responses in nonresponder mice primed with GAT-methylated albumin (11). GAT-TsF is a protein that has antigen-binding activity and determinants encoded by the *I-J* subregion of the MHC but no determinants encoded by immunoglobulin heavy or light chain constant region genes (12). Germain *et al.* (13) have demonstrated that GAT-TsF crossreacts with idiotypic determinants expressed by certain murine anti-GAT antibodies.

To obtain sufficient quantities of GAT-TsF for biochemical and genetic analysis, T cell hybridomas were constructed by fusing partially purified T cells from GAT-primed, nonresponder DBA/1 (*H-2^q*) mice with the AKR (*H-2^k*) thymoma, BW5147. The prototype clone that was selected for study (258 C4.4) has been described (14). Recently, we have reported on the purification of GAT-TsF released into the culture medium by 258 C4.4 cells to chemical homogeneity (15).

The development of a hybridoma that secretes GAT-TsF increases the quantity of mRNA available that ultimately can be used to clone the gene(s) responsible for the synthesis of this factor. In this communication, we report the isolation and partial purification of the GAT-TsF mRNA from 258 C4.4. This mRNA has been translated by using a rabbit reticulocyte lysate system and the *in vitro* translated product has biological activity.

MATERIALS AND METHODS

Materials. GAT was purchased from Vega–Fox; oligo(dT)-cellulose was purchased from P-L Biochemicals; rabbit reticulocyte *in vitro* translation kit and sucrose were obtained from Bethesda Research Laboratories; Sepharose was purchased from Pharmacia; Lichrosorb RP-8 was obtained from E. Merck (Darmstadt, Federal Republic of Germany).

Antisera. Alloantisera having specificities for the *I^q* or *I-J^s* subregions in the MHC were prepared by Donald Shreffler, Washington University School of Medicine. Briefly, anti-*I^q* antisera were prepared by injecting (AQR × A)F$_1$ mice with lymphocytes from B10.T (6R) mice. Anti-*I-J^s* antisera were made by immunizing [A.TL × B10.S (9R)]F$_1$ mice with B10.HTT lymphocytes.

Cells. A GAT-TsF secreting hybridoma was made by fusion of DBA/1 splenic suppressor T cells with the hypoxanthine phosphoribosyltransferase (HPRT$^-$) AKR thymoma BW5147 (14). The prototype hybridoma (258 C4.4) or BW5147 cells were grown to maximum density at 37°C in a 5% CO_2/95% air atmosphere at which time the cells were harvested and stored at −70°C.

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Abbreviations: GAT, poly(LGlu^{60}LAla^{30}LTyr^{10}); HPRT, hypoxanthine phosphoribosyltransferase; MHC, major histocompatibility complex; TsF, T cell suppressor factor; GAT-TsF, GAT-T cell suppressor factor; GAT-methylated albumin, GAT complexed with methylated bovine serum albumin; SRBC, sheep red blood cells; PFC, plaque-forming cell(s); S_{50}, 50% suppression of the PFC or T cell proliferative responses.

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Affinity Chromatography. Sepharose-immobilized mouse anti-I^q and anti-$I\text{-}J^s$ antisera were prepared by coupling heat-inactivated alloantisera by cyanogen bromide-activated Sepharose 4B as described (10). *In vitro* translated material (30 μl) was incubated with 50 μl of Sepharose-conjugated anti-I^q or anti-$I\text{-}J^s$ antisera for 2 hr at room temperature. The gel was washed extensively in a cotton-plugged Eppendorf pipette tip with phosphate-buffered saline. Bound proteins were eluted with 40% 1-propanol in H_2O and then lyophilized. This material was reconstituted to 0.1 ml with phosphate-buffered saline containing bovine serum albumin at 1 mg/ml and 20% 1-propanol and assayed for GAT-TsF activity.

mRNA Isolation and Cell-Free Translation. Approximately 2×10^9 ($\approx$3 ml of packed cells) hybridoma cells were lysed in 30 ml of NET buffer (0.1 M NaCl/5 mM EDTA/20 mM Tris·HCl, pH 8.0, containing 0.5% $NaDodSO_4$). Thirty milliliters of Tris-saturated phenol was added to the cells and mixed, and the aqueous and organic phases were separated. The organic phase was reextracted with 30 ml of NET buffer and both aqueous phases were pooled and adjusted to 1.0 M NaCl by adding NaCl crystals. The pooled aqueous phases were mixed with 1 vol of chloroform/isoamyl alcohol (22:1). The mixture was agitated and centrifuged, and 0.1 vol of 2 M sodium acetate (pH 5.7) and 2 vol of ethanol were added to the aqueous phase ($\approx$60 ml). After precipitation with ethanol overnight, the precipitate was collected by centrifugation at 15,000 $\times$ *g* for 15 min and resuspended in 110 ml of H_2O to which 110 ml of 2$\times$ KET buffer (0.1 M KCl/5 mM EDTA/20 mM Tris·HCl, pH 8.0) was then added. To the 220-ml volume, 500 mg of oligo(dT) was added and gently shaken for 15 min. The oligo(dT)-cellulose was separated from the supernatant by centrifugation and washed twice with NET buffer, and bound material was then eluted with 10 ml of 10 mM Tris/5 mM EDTA. This oligo(dT)-binding procedure was performed twice on the 220-ml ethanol-precipitated material. The Tris/EDTA eluted material was pooled, solid KCl was added to give a final concentration of 0.5 M, and the mixture was passed over an oligo(dT) column. Material bound to the column was washed with KET, eluted with 6 ml of H_2O, brought to 0.2 M potassium acetate, and precipitated with ethanol. Approximately 675 μg of poly(A)$^+$mRNA representing $\approx$3% of the total nucleic acid pool in the first ethanol precipitate was obtained by this procedure.

The mRNA from the hybridoma 258 C4.4 and the BW5147 cell line was translated in a rabbit reticulocyte lysate system as originally reported by Pelham and Jackson (16) by using a commercially available *in vitro* translation kit.

Sucrose Density Gradient Centrifugation of mRNA. Hybridoma 258 C4.4 and BW5147 mRNA (130 μg) were fractionated on 15–30% sucrose density gradients in 40-ml Beckman Quick Seal polyallomer tubes by using sterile 20 mM sodium acetate (pH 6.0) containing 0.5% $NaDodSO_4$ as the buffer system. The tubes were centrifuged for 2 hr and 15 min at 20°C in a vTi50 rotor at 45,000 rpm. After centrifugation, 1.5-ml fractions were collected, the absorbance at 260 nm was determined, and each fraction was then precipitated with ethanol three times. The ethanol precipitates were lyophilized and reconstituted in 0.2 ml H_2O.

HPLC. *In vitro* translated products that bound to Sepharose-anti-I^q were further purified by reverse-phase HPLC by using a Lichrosorb RP-8 resin in a 4.5 $\times$ 250 mm column. Chromatography was performed by using a 1.0 M pyridine/0.5 M acetate, pH 5.5, buffer system and a 0–60% 1-propanol step-gradient. The flow rate was 20 ml/hr and 1-ml fractions were collected. The following step-gradient program was used: 15 min at 0% 1-propanol, 30 min at 15% 1-propanol, 60 min each for 30% and 40% 1-propanol, 24 min each for 45% and 60% 1-propanol. Fractions were then lyophilized and prepared for analysis of GAT suppressor activity.

Electrophoresis. After purification with immunoadsorbent and HPLC, *in vitro* translated GAT-TsF was electrophoresed on an 8–18% gradient $NaDodSO_4$-containing polyacrylamide gel under nonreducing conditions by using the buffer system described by Laemmli (17). Purified factor equivalent to 70 μl of *in vitro* translated protein was lyophilized and run in the gel at 125 V for 7 hr. After electrophoresis, 4-mm slices were cut and washed once with 1 ml of RPMI-1640 medium containing bovine serum albumin at 1 mg/ml on a rotator for 2 hr to lower the concentrations of $NaDodSO_4$ and other soluble gel constituents. The slices were then mashed with a Teflon rod and incubated in 1 ml of phosphate-buffered saline containing bovine serum albumin at 1 mg/ml on a rotator. After 8 hr, the supernatant fluids were collected and each mashed slice was again washed with phosphate-buffered saline containing bovine serum albumin. Supernates for each elution were pooled for each fraction, lyophilized, and prepared for assay of suppressor activity.

Assay for the GAT-Specific Suppressor Factor. *In vitro* translated products were assayed for the presence of the GAT-specific suppressor factor by using a T cell proliferation assay or *in vitro* PFC response by spleen cells (or both). The proliferation assay was performed as described (6). Briefly, DBA/1 or B10.Q ($H\text{-}2^q$) mice were immunized in the footpad with GAT at 5–20 μg in the form of a GAT-methylated albumin complex in complete Freund's adjuvant. After 12–14 days, single cell suspensions were prepared from popliteal lymph nodes. The T cells were enriched by filtration through nylon wool columns (18) and cultured in flat bottom microtiter plates at 4×10^5 cells per well in RPMI-1640 medium supplemented with 10% fetal calf serum, 20 mM Hepes, 0.01 mM 2-mercaptoethanol, and antibiotics. Incorporation of thymidine into DNA was measured on day 5 after a 24-hr pulse with [^{3}H]thymidine. The *in vitro* splenic PFC responses to GAT-methylated albumin or sheep red blood cells (SRBC) by using modified Mishell–Dutton conditions were performed as described (19). In some experiments, the data have been expressed as the reciprocal of the dilution of the translation mixture that causes 50% suppression of the PFC or the T cell proliferative responses (S_{50} units/ml).

RESULTS

Analysis of the Biological Activity of Proteins Synthesized by Using Sucrose Density Gradient-Fractionated mRNA from 258 C4.4 and BW5147. Total poly(A)$^+$mRNA from the hybridoma, 258 C4.4, and the thymoma, BW5147, were fractionated on sucrose density gradients as described. The mRNA from each fraction was translated in a rabbit reticulocyte lysate translation system and the *in vitro* translated proteins were assayed for GAT-TsF activity. Preliminary studies showed that the *in vitro* translation of unfractionated mRNA extracted from 258 C4.4 and BW5147 contained mRNA molecules that code for suppressor proteins. Subsequent fractionation of 258 C4.4 mRNA on a sucrose density gradient resulted in the separation of mRNAs that code for the GAT-specific T cell suppressor factor and an unidentified nonspecific suppressor factor (Table 1). The data in Table 1 compare pooled fractions from sucrose density gradient-fractionated 258 C4.4 and BW5147 mRNAs in terms of the ability of their respective *in vitro* translated products to suppress T cell proliferation stimulated by GAT or GAT-methylated albumin. The proteins translated from the mRNA fractions 13–15 from 258 C4.4 specifically suppressed DNA synthesis by DBA/1 lymph node T cells stimulated by GAT but not DNA synthesis in cultures stimulated by GAT-methylated

albumin. No suppressive activity was obtained from mRNA fractions 13–15 obtained from BW5147. The proteins encoded by the mRNA fractions 19–21 from both 258 C4.4 and BW5147 suppressed T cell proliferation regardless of the stimulating antigen. Fig. 1 shows the capacity of different size classes of mRNA translated *in vitro* to code for proteins that suppress the GAT-specific antibody responses. The size of the message that codes for GAT-TsF appears to be 16 S, whereas the size of the message coding for the nonspecific suppressor corresponds to 10 S. Fractions 18, 19, and 20 contained this nonspecific suppressive activity but the quantity was not determined.

Recognition of *in Vitro* Translated GAT-TsF by Anti-I^q Antiserum. Fractionated mRNA coding for GAT-TsF (fraction 14 from 258 C4.4) was translated *in vitro* and tested for binding to anti-I^q or anti-I-J^s alloantisera. We have previously shown that the anti-I^q—but not the anti-I-J^s—antibodies bind to GAT-TsF produced by 258 C4.4 (14). BW5147 mRNA of the equivalent size was also translated *in vitro* and tested for any suppressor activity before and after adsorption to these antisera. The translated products from the 16S mRNA from 258 C4.4 and BW5147 were subjected to affinity chromatography on Sepharose-immobilized anti-I^q or anti-I-J^s antisera. GAT-TsF activity was not adsorbed by anti-I-J^s antiserum, whereas anti-I^q antiserum bound the activity (Fig. 2). The data in Fig. 2, which are presented in terms of the quantity of GAT-TsF (in S_{50} units) passing through the immunoadsorbent, show that the amount of GAT-TsF in the anti-I-J^s column effluent was similar to the unadsorbed *in vitro* translated GAT-TsF. Sepharose-bound anti-I^q antiserum removed all detectable GAT-TsF activity.

The data presented above represent GAT-TsF activity in terms of suppression of the *in vitro* anti-GAT antibody response. In addition, *in vitro* translated GAT-TsF suppressed GAT-induced T cell proliferation. Table 2 shows that anti-I^q antiserum—but not anti-I-J^s antiserum—bound GAT-TsF and prevented suppression of the GAT-induced proliferative response. *In vitro* translated GAT-TsF can be eluted from the Sepharose-anti-I^q affinity adsorbent by using a 40% 1-propanol wash. However, we have repeatedly observed that elution from these affinity adsorbents with 1-propanol results in detectable levels of nonspecific suppression. Although this effect is readily observed in the case of the BW5147-translated products that were eluted with 40% 1-propanol, we do not know the mechanism responsible for the generation of this nonspecific activity. However, this could be a result of the toxicity of residual propanol remaining in the diluted samples.

Table 1. Suppressor activities of *in vitro* translated proteins made from sucrose density gradient-fractionated mRNA from 258 C4.4 and BW5147

Source of mRNA	Fraction no.	[^{3}H]Thymidine incorporation, cpm*		
		Medium	GAT	GAT-methylated albumin
(No factor)	—	4,117	20,015	47,313
BW5147	10–12	4,311	18,140	45,408
BW5147	13–15	4,280	15,790	48,909
BW5147	16–18	4,870	15,775	43,808
BW5147	19–21	5,979	8,552	29,662
258 C4.4	10–12	3,997	22,561	38,892
258 C4.4	13–15	5,661	7,726	47,110
258 C4.4	16–18	3,955	19,557	47,505
258 C4.4	19–21	7,654	8,720	28,322

* Thymidine incorporation by lymph node T cells from mice primed with GAT-methylated albumin in the presence or absence of *in vitro* translated proteins from pooled fractions of sucrose density gradient-fractionated hybridoma mRNA or BW5147 mRNA. The final dilution of each translated fraction was 1:3,600. Fractions 1–9 had no suppressor activity.

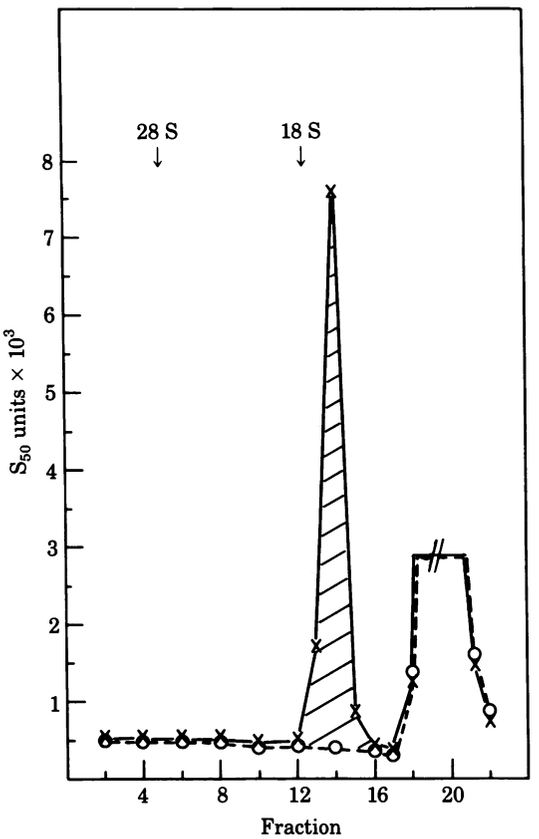

FIG. 1. Quantitation of GAT-TsF activity in the translation products from sucrose density gradient-fractionated 258 C4.4 and BW5147 mRNA. The mRNA from 258 C4.4 or BW5147 was fractionated on 5–20% sucrose gradients and translated *in vitro*, and the products were assayed for suppression of GAT-specific PFC responses. The data describe the suppressor activity of the *in vitro* translation products of 258 C4.4 mRNA (×) and BW5147 (○). Nonspecific suppression was observed in fractions 19, 20, and 21, but these fractions were not titrated; therefore, suppression is indicated but no estimate of quantity has been made.

Isolation of GAT-TsF by Reverse-Phase HPLC. Because these immunoadsorbents appear to bind other proteins as well as GAT-TsF, we further purified the *in vitro* translated GAT-TsF that had been bound to and eluted from Sepharose-anti-I^q by reverse-phase HPLC. GAT-TsF activity was eluted from the Lichrosorb RP-8 resin with 40% 1-propanol when a 0–60% 1-propanol step-gradient was used (Fig. 3). The suppressor activity eluted in a single fraction with recovery of virtually all of the applied activity; <10% suppression was observed in all other fractions. This preparation of affinity-purified and chromatographically purified GAT-TsF was analyzed on an 8–18% polyacrylamide gradient gel containing $NaDodSO_4$ under nonreducing conditions. After the elution of protein from 4-mm gel slices, supernatant fluids were assayed for specific suppressor activity. We have found that $NaDodSO_4$ does not inactivate GAT-TsF activity and that dilution of the $NaDodSO_4$ (by several thousandfold) is sufficient to abrogate its toxicity for cultured lymphocytes. The major peak of suppressor activity was found in a slice corresponding to an apparent M_r 19,000 (Fig. 4). This band specifically inhibited the GAT-specific antibody response *in vitro* and also the GAT-induced T cell proliferative response (data not shown).

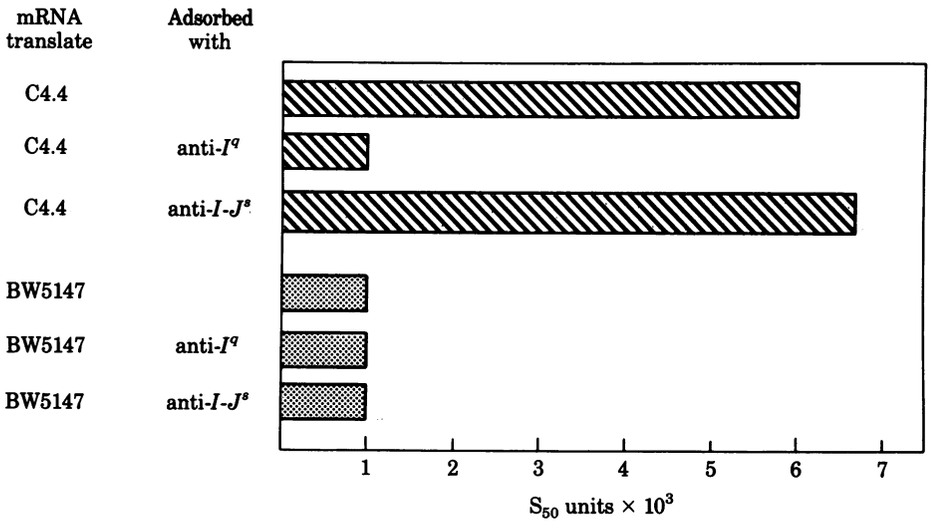

FIG. 2. Binding of *in vitro* translated GAT-TsF activity by anti-I^q immunoadsorbents. Fractionated mRNA from 258 C4.4 (fraction 14) and from BW5147 (fractions 13–15) was translated *in vitro* and filtered through Sepharose-bound anti-I^q or anti-*I*-J^s antisera. The suppressive activity was quantitated by using the PFC assay and the data are reported as the number of S_{50} units remaining in the supernatant after adsorption with the immobilized antisera. None of these translation products inhibited immune responses to SRBC. The lowest dilution of translated protein that was tested was 1:1,000; thus activity ≤1,000 S_{50} units signifies no detectable suppression.

DISCUSSION

In vitro translation of fractionated mRNA isolated from 258 C4.4—a T cell hybridoma producing the GAT-specific suppressor factor, GAT-TsF—resulted in the biosynthesis of two classes of immunosuppressive proteins, one of which was specific for the immune response to GAT and the other of which was a nonspecific suppressor. The nonspecific suppressor protein was encoded by 10S mRNA and was found also in the BW5147 AKR thymoma that was used in the fusion with the GAT-TsF-secreting suppressor T cell. This nonspecific suppressor factor was able to inhibit T cell proliferative responses to GAT and GAT-methylated albumin by GAT-methylated albumin-primed T cells. Furthermore, the nonspecific suppressor protein suppressed the *in vitro* anti-GAT and anti-SRBC antibody responses.

GAT-TsF that was translated from the 16S mRNA possessed determinants encoded by the *I* subregion of *H-2* as is the case for the secreted GAT-TsF (14) and GAT-TsF extracted from lymphoid organs or cells from GAT-immunized mice (9, 12). Like the GAT-TsF secreted by the hybridoma, GAT-TsF translated *in vitro* adsorbs to a reverse-phase chromatographic column and is eluted with 40% 1-propanol. The *in vitro* translated GAT-TsF had an apparent M_r of 19,000, which is smaller than the hybridoma-secreted protein of M_r 24,000 (15). Because little, if any, glycosylation occurs in the rabbit reticulocyte lysate translation system (20, 21), the size difference between the secreted GAT-TsF and the *in vitro* translated form is most likely due to a difference in carbohydrate content of the two forms of GAT-TsF. Indirect evidence that GAT-TsF is a glycoprotein is derived from the observation that GAT-TsF secreted by the hybridoma 258 C4.4 is bound by the immobilized carbohydrate-specific lectins, peanut agglutinin and lentil lectin (unpublished observations).

The fact that unprocessed *in vitro* translated GAT-TsF can suppress both T cell proliferation and the appearance of GAT-specific antibody-producing cells indicates that the biological function of this protein is not strictly dependent on the presence of carbohydrate or other posttranslational modifications (or both) because—as stated above—the reticulocyte-translating system is essentially devoid of glycosylating activity (20, 21). Furthermore, the ability of anti-I^q antiserum to bind the *in vitro* translated form of GAT-TsF strongly implies that the *Ia* determinants on GAT-TsF that are recognized by this alloantiserum are contained in the primary amino acid sequence as are determinants encoded by the *I-A* and *I-E* subregions (22) and are not carbohydrate as reported for some other *Ia* antigens (23, 24).

It is difficult to calculate the quantity of GAT-TsF that is produced *in vitro* because its specific activity may be different from the secreted material. This calculation is made even more difficult because the number of reinitiation events for translation of the factor is not known. Rough calculations—by using

Table 2. Specificity of mouse anti-I^q antisera for *in vitro* translated GAT-TsF activity in suppressing the GAT proliferative response

In vitro translation products*	Sepharose-bound immunoadsorbent	% suppression of GAT proliferative response at 1:5,000 dilution	
258 C4.4 *k/q*	—	84	
BW5147 *k*	—	0	
		Effluent	Eluate†
258 C4.4	anti-I^q	3	88
258 C4.4	anti-*I*-J^s	93	50
BW5147	anti-I^q	10	36
BW5147	anti-*I*-J^s	0	30

* *In vitro* translated proteins were made by using sucrose density gradient-fractionated mRNA from 258 C4.4 (fraction 14) and BW5147 (fractions 13–15).

† Proteins that were bound to the Sepharose-conjugated antisera were eluted with 40% 1-propanol.

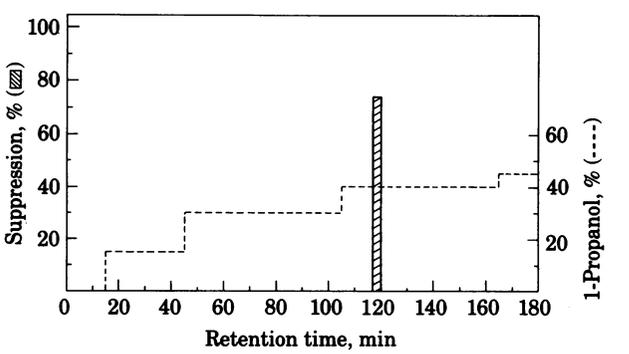

FIG. 3. Purification of *in vitro* translated GAT-TsF by reverse-phase HPLC. 16S mRNA (fraction 14 from the sucrose density gradient) from 258 C4.4 was translated *in vitro*, adsorbed with Sepharose-anti-I^q, eluted with 40% 1-propanol, and further purified by reverse-phase HPLC by using a propanol elution step-gradient (----). Fractions were assayed for GAT-TsF activity in suppressing the GAT-specific PFC response *in vitro*. All fractions were tested for suppressive activity but only levels of suppression >20% are recorded.

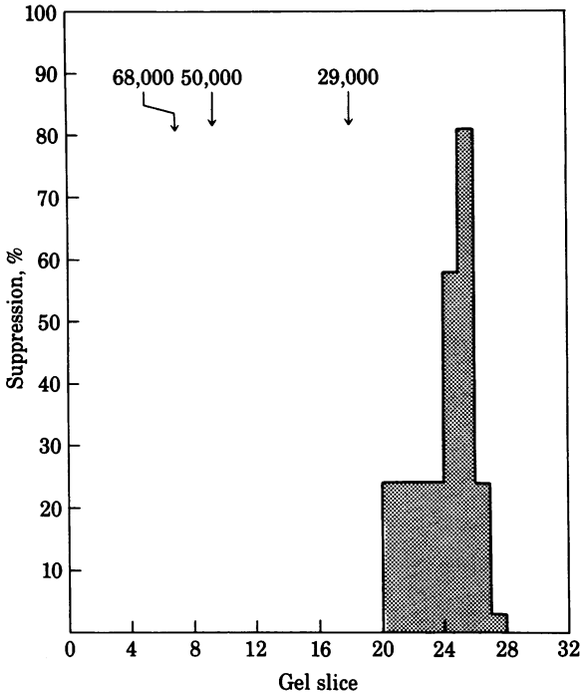

FIG. 4. M_r estimates by $NaDodSO_4$/polyacrylamide gel electrophoresis of HPLC-purified GAT-TsF. *In vitro* translated GAT-TsF that was further purified by HPLC was subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis by using an 8–18% acrylamide gradient. GAT-TsF activity from gel slices was determined by inhibition of the GAT-specific PFC response. Data reported are expressed as the percent suppression of the anti-GAT antibody response at a 1:5,000 dilution of material extracted from the gel slices. M_r markers are the G protein (70,000), N protein (50,000), and M proteins (29,000) from vesicular stomatitis virus.

the specific activity of the secreted form of GAT-TsF as 7.8×10^4 S_{50} units/ng of protein (15)—indicate that the quantity of *in vitro* translated GAT-TsF shown in Figs. 1 and 2 represents a low efficiency of GAT-TsF mRNA translation. However, it is possible that the *in vitro* translated form of GAT-TsF has a much lower specific activity as a result of a lack of posttranslational modifications and that the efficiency of translation may be higher than that observed. It should be noted that posttranslational modifications of GAT-TsF could be important *in vivo* for protein stability and homing. Related to these problems is the fact that amino acid analysis of GAT-TsF shows it to be low in methionine (15). For this reason, initial experiments that used [^{35}S]methionine did not yield a product in the range of M_r 19,000 with a sufficient amount of radioactivity for use in studies of rates of synthesis.

It is interesting that a M_r 19,000, biologically active GAT-TsF can be produced by using a pool of 16S mRNA. mRNA of this size could encode for proteins as large as M_r 50,000–60,000. At the present time we are unsure whether the GAT-TsF isolated from the *in vitro* translation system represents the only product of its message or whether it represents an enzymatic cleavage product of a larger precursor molecule. It should be noted in this regard that we could find no evidence for a larger biologically active moiety. It is possible that a larger precursor exists that is biologically inactive. Eluates from GAT-Sepharose affinity columns did contain proteins of up to M_r 70,000; however, at least some of these proteins were specified by mRNA from the parental cell line, BW5147, as well as the hybridoma (data not shown). Thus, the question of a possible relationship between this suppressor molecule and other larger suppressor factors [reported by other groups (25, 26)] is unresolved at this time. However, factors from cells operating at different points in the suppressor cell system may have different structures. The fact remains that the GAT-TsF obtained by *in vitro* translation has all of the biological and serological properties of GAT-TsF secreted by the T cell hybridoma and GAT-TsF obtained from tissue extracts. This suggests that its biologically active form need be no larger than M_r 19,000.

The logical extension of this work is to clone the genes responsible for the synthesis of GAT-TsF as well as other immunoregulatory molecules by making cDNA libraries from their respective mRNAs. Such studies should enhance our understanding of the nature of the suppressor molecules encoded by the *I-J* subregion of the MHC.

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