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Molecular Weight Determination of Two Genetically Linked Cell Surface Murine Antigens: ThB and Ly-6

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Abstract. Various murine tumor lines were screened by FACS analysis for the surface antigens ThB and Ly-6.2. Positive cell lines were used for immunoprecipitation studies. A monoclonal ThB-specific antibody immunoprecipitated a unique acidic protein of approximately 16 000 daltons from several positive tumors and from concanavalin A (Con-A) and LPS activated splenic lymphocytes. Monoclonal Ly-6.2-specific antibody was used to immunoprecipitate a 33 500 dalton protein that was shown to exist in four similarly sized forms with different basic charges. In the course of these studies, the apparent molecular weight of the surface antigen T 30, immunoprecipitated with a monoclonal T 30-specific antibody from the cell line EL4, was found to be approximately 25 000 daltons.

Introduction

The morphological identity of different subsets of lymphocytes has led to attempts to correlate lymphocyte functions and ontogeny with cell surface antigens. Several discrete determinants have now been described since the earlier identification of Thy-1, Lyt-1, Lyt-2, and Lyt-3 as T-cell markers and surface Ig as a B-cell marker (for review see McKenzie and Potter 1979). In the course of these studies, an interesting locus was discovered, which controls expression of the surface markers Ly-6 (McKenzie et al. 1977), H 9/25 (Takei et al. 1980) [and possibly Ly-8 (Frelinger and Murphy 1976), Ala-1 (Feeny and Hämmerling 1976), and Dag (Sachs et al.

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1973), though these may have identity with Ly-6] and regulates the level of ThB on B cells (Eckhardt and Herzenberg 1980). The genetic linkage between high levels of ThB expression and the Ly-6.2 allele, with no recombinants in 96 backcross mice (Eckhardt and Herzenberg 1980), raised the possibility of a relationship between the two antigens. Work in our laboratory using monoclonal antibodies and FACS analysis enabled us to determine the cellular distribution of these two antigens, both in relation to different tissues and to other lymphocyte markers (Eckhardt and Herzenberg 1980). The presence of Ly-6 on most peripheral T-cells and B-cells, but only on the cortisone resistant thymic population, contrasted with the ThB distribution on B cells and 50% of thymocytes, strongly suggesting that the two antigens were separate. Until recently, attempts to further clarify this by chemical characterization of Ly-6.2 and ThB antigens derived from spleen cells and thymocytes were unsuccessful. However, we now report studies on the molecular nature of these antigens, which we have immunoprecipitated from various cell lines and also from normal blast cells.

Materials and Methods

Cell lines. The cell lines used in these studies are listed in Table 1. They were maintained in RPMI 1640 (Grand Island Biological Co., Grand Island, New York) supplemented with 15% fetal calf serum and antibiotics. Mercaptoethanol at 1×10^{-5}M final dilution was added for culture of the 5F10 and CH1 cell lines.

Monoclonal antibodies used for immunoprecipitations. Monoclonal anti-Ly-6.2 (S8.106) (Kimura et al. 1980) was kindly donated by Dr. U. Hämmerling of the Sloan-Kettering Cancer Center. This antibody is of the mouse IgG 2a subclass. Monoclonal anti-ThB (53–9.2) was derived in this laboratory (Eckhardt and Herzenberg 1980). The latter is of the rat IgG 2c subclass and because of the putative property of nonspecific binding with this class of antibody, a second rat IgG 2c monoclonal antibody (53–8.1) against the antigen T 30 (Ledbetter and Herzenberg 1979) was used in these studies.

Immunofluorescence of cell suspensions and FACS analysis. Immunofluorescence staining with anti-ThB was performed using fluorescein-conjugated antibodies. Fluorescein conjugations were with fluorescein isothiocyanate. An indirect system was used for immunofluorescence staining with anti-Ly-6.2. The cells

Table 1. The cell lines used for immunoprecipitation of ThB and Ly-6.2 and their relative antigen densities

Cell line	Type	Strain of origin	Relative mean fluorescence* of cells strained with:	
			ThB	Ly-6.2
Wehi 222	T Lymphoma	ATL	2.74	–
GRSL	Spontaneous leukaemia: pre T cell	GR	2.10	–
CH1	B cell lymphoma	B10.A	2.92	–
EL4	T cell lymphoma	C57BL/6	–†	2.22
Bw5147	T cell lymphoma	AKR	–	2.49
5F10	Hybrid T cell line		–	2.80

* The geometric mean fluorescence values of the fluoresceinated cells (see *Materials and Methods*) in standard arbitrary logarithmic units.

† Denotes absence of antigen.

were first incubated with monoclonal anti-Ly-6.2, washed, and fluorescein conjugated monoclonal anti-Igh-1a (21–74.4) (Oi and Herzenberg 1979) was added as second step reagent. All antibodies were centrifuged at 100000 g before use; 2.5×10^5 tumor cells in RPMI 1640 containing 1% fetal calf serum and 0.1% NaN_3 were stained on ice in microtiter wells with saturating levels of directly fluoresceinated first and second step reagents as previously described (Rogers and Matossian-Rogers 1981). The cells were analyzed using a modified FACS II (Becton Dickinson FACS systems, Mountain View, California) fitted with a logarithmic amplifier covering a nearly 10^4 fold range of fluorescence intensity.

Biochemical procedures. Cells were surface labeled with ^{125}I by the lactoperoxidase technique as previously described (Ledbetter et al. 1981) with the minor modifications of using $3–4 \times 10^7$ tumor or blast cells in 200 µl of phosphate buffered saline (PBS), 1.5 mCi ^{125}I (Amersham Corp., Arlington Heights, Illinois) and pulsing with H_2O_2 at 2 min intervals. Cells were washed twice and extracted in 1.5 ml of lysis buffer (0.5% Nonidet-P40, Particle Data, Elmhurst, Illinois; 50 mM Tris, 150 mM NaCl, 0.2% NaN_3, 5 mM ethylenediamine tetraacetic acid (EDTA) and 50 mM phenyl methyl sulfonyl fluoride (PMSF) for 30 min at 4°. Nuclei were removed by centrifugation at 12000g for 10 min. Sodium deoxycholate and SDS were then added to the supernatant to give final concentrations of 1% and 0.1%, respectively. Biosynthetic labeling with ^{35}S methionine (Amersham Corp., Arlington Heights, Illinois) and ^{35}S cysteine (New England Nuclear, Boston, Massachusetts) was carried out by incubating cells at 4×10^6/ml (for approximately 18 h at 37° in a humidified 7% CO_2 in air atmosphere) in methionine and cysteine free RPMI 1640 medium that contained 200 µCi/ml of each labeled amino acid. Cells were solubilized as described above.

Immunoprecipitation and polyacrylamide gel electrophoresis. The monoclonal antibodies used for immunoprecipitates were of the IgG2a (mouse) and IgG2c (rat) subclasses, which bind directly to protein A. Extracts from 10^7 blast or tumor cells were precleared with 200 µl of 10% fixed *S. aureus* Cowan 1 strain (SAC) organisms (The Enzyme Center, Boston, Massachusetts) and immunoprecipitated with 5 µg of antibody. The antigen-antibody complexes were removed by the addition of 15 µl SAC. The *S. aureus* bound complexes were then washed and eluted as previously described (Jones 1980). For the first dimension, nonequilibrium pH gradient electrophoresis was used and for the second dimension or for the one dimension only 12.5% SDS polyacrylamide slab gels were used.

Molecular weight markers (Pharmacia Fine Chemicals, Pistcataway, New Jersey) run on each gel were visualized by staining with Coomassie Brilliant Blue (Bio-Rad Laboratories, Richmond, California). Autoradiography of gels with ^{35}S labeled proteins was with Kodak No-Screen film (Eastman Kodak Co., Rochester, New York) at room temperature and gels with ^{125}I labeled proteins were exposed to Kodak X-omat R film at −70° with Dupont Cronex Lightning-Plus intensifying screens (DuPont Instruments, Wilmington, Delaware).

Results

A number of tumor cell lines were screened for the ThB antigen using directly fluorescein-conjugated ThB-specific antibody and FACS analysis. The positive cell lines that were used for the immunoprecipitation studies are shown in Table 1 together with their respective mean fluorescence values on anti-ThB staining; these are indicative of the relative densities of surface ThB antigen. The CH1 tumor had the highest density of ThB and was the cell line from which the ThB antigen was consistently the most efficiently labeled. Accordingly, the FACS staining profile and the representative one dimensional gel autoradiographs are shown for this tumor (Figs. 1 and 2). The discrete band shown in Figure 2a was obtained from an anti-ThB immunoprecipitate of CH1 cells iodinated by the lactoperoxidase catalysis method and run in one dimension electrophoresis under nonreducing conditions on a 12.5% polyacrylamide gel. It indicates that the apparent molecular weight of ThB is approximately 16000. The same result was obtained from ^{125}I labeled immunoprecipitates of all of the other ThB positive tumors used and from CH1 (Fig. 2b) and

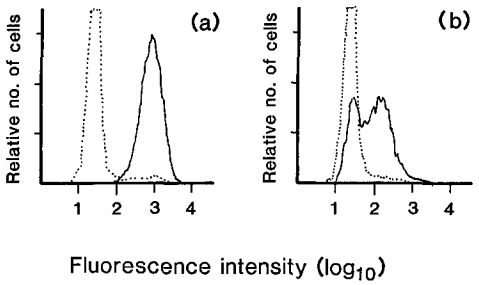

Fig. 1. Immunofluorescence staining of (a) CH1 tumor cells with fluorescein-conjugated anti-ThB (——) and (b) EL4 tumor cells with anti-Ly-6.2 and fluorescein conjugated anti-Igh1a as second step reagent. The *dotted line* in (a) represents autofluorescence of CH1 cells and in (b) background staining of EL4 cells with second step alone; the cells were analyzed with a FACS II fitted with a logarithmic amplifier.

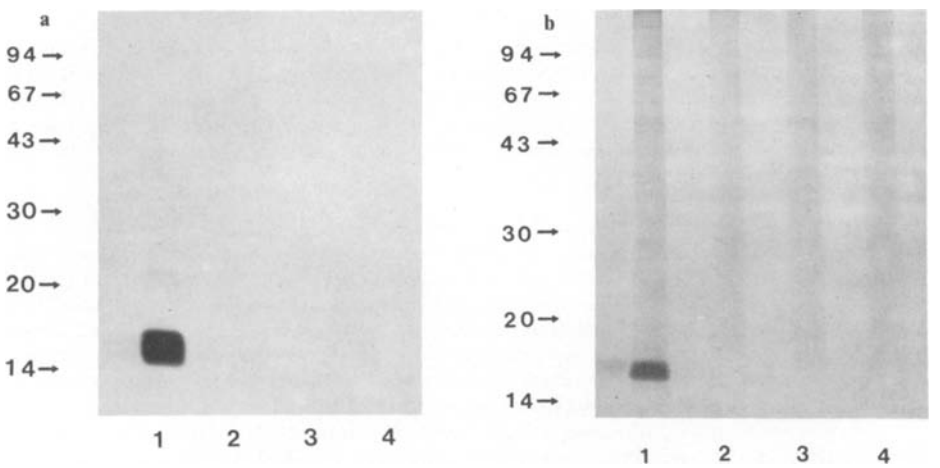

Fig. 2a and b. Immunoprecipitation of antigens derived from CH1 tumor cells labeled with ^{125}I (**a**) or ^{35}S (**b**). The antigens were immunoprecipitated using (1) anti-ThB (53–9.2), (2) anti-Ly-6.2 (S8.106), (3) anti-T.30(53–8.1).(4) is background (*S. aureus* alone). Immunoprecipitates were run on 12.5% polyacrylamide gels under nonreducing conditions.

all other positive tumors when immunoprecipitates were derived from cells metabolically labeled by the incorporation of ^{35}S cysteine and methionine. The 16K band was also immunoprecipitated from ^{125}I labeled LPS (Fig. 3) and concanavalin A (Con-A) activated C57BL/6 splenic lymphocytes. All the monoclonal antibodies used showed some degree of nonspecific binding to labeled antigens derived from activated cells, as indicated by the common bands in lanes 1, 2, and 3 of Figure 3. However, the 16K band was always uniquely precipitated by anti-ThB from positive cell lines but not from ThB negative cells (see Fig. 5a, b).

Immunoprecipitates from the ^{125}I labeled CH1 tumor were used to show that the ThB antigen is a single polypeptide chain since the apparent molecular weight was unchanged under reducing or nonreducing conditions. On two dimensional electrophoresis the ThB antigen appears as a unique acidic protein (Fig. 4).

The Ly-6.2 antigen was also immunoprecipitated from a number of tumor lines (Table 1), but for this antigen, there was no apparent relationship between relative antigen density as determined by the mean fluorescence intensity and ease of

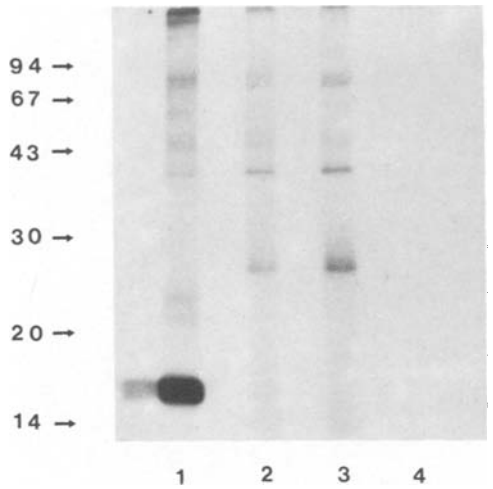

Fig. 3. Immunoprecipitation of ^{125}I labeled antigens derived from LPS activated splenocytes. The antigens were immunoprecipitated with (1) anti-ThB (53–9.2), (2) anti-La-6.2 (S8.106), (3) anti-T.30 (53.8.1), (4) is background (*S. aureus* alone). Immunoprecipitates were run on 12.5% polyacrylamide gels under nonreducing conditions.

Fig. 4. Two dimensional gel electrophoresis of the ThB antigen immunoprecipitated from ^{125}I labeled CH1 tumor cells using anti-ThB (53.9.2). The first dimension separation was by nonequilibium pH-gradient electrophoresis (acidic proteins on the right). The second dimension separation (top to bottom) was by SDS-PAGE on 12.5% gels.

precipitation of ^{125}I labeled antigen. The cell line EL4 had the lowest density, but consistently gave the best yield of ^{125}I labeled immunoprecipitate; accordingly, the staining profile and autoradiographs for the Ly-6.2 antigen shown in Figures 1b and 5, respectively, were obtained from this tumor. Figure 5a shows a 33 500 MR moiety specifically precipitated from ^{125}I labeled EL4 tumor cells by monoclonal Ly-6.2-

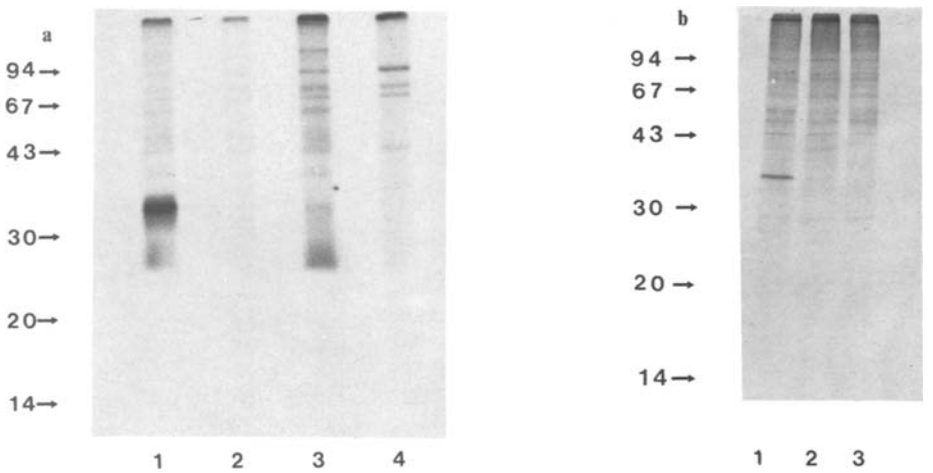

Fig. 5a and b. Immunoprecipitation of antigens derived from EL4 tumor cells labeled with ^{125}I (**a**) or ^{35}S (**b**). In **a** the antigens were immunoprecipitated using (1) anti-Ly-6.2 (S8.106), (2) anti-ThB (53–9.2), (3) anti-T 30 (53–8.1), and (4) background (*S. aureus* alone). In **b** immunoprecipitates were with (1) anti-Ly-6.2 (S8.106), (2) anti-T 30 (53.8.1), and (3) anti-ThB (53.9.2). Immunoprecipitates were run on 12.5% polyacrylamide gels under nonreducing conditions (**a**) and reducing conditions (**b**).

specific antibody. This gel was run under nonreducing conditions. However, the apparent molecular weight was unchanged under reducing conditions; thus, the Ly-6.2 antigen is a single polypeptide chain. A similar 33 500 MR band was obtained in trace amounts from ^{125}I labeled cells of the BW5147 and 5F10 cell lines. We were unable to immunoprecipitate Ly-6.2 antigen from ^{125}I labeled blast cells obtained from splenic lymphocytes on activation with either LPS or Con-A. Furthermore, Ly-6.2 negative cell lines did not give rise to the 33.5K band (see Fig. 2a, b).

All the Ly-6.2 positive tumors yielded quantitatively similar amounts of anti-Ly-6.2 immunoprecipitate when labeled metabolically with ^{35}S cysteine and methionine. Fig. 5b shows the 33 500 MR band obtained from ^{35}S labeled anti-Ly-6.2 immunoprecipitates by one dimensional electrophoresis under reducing conditions. Some nonspecific precipitation of labeled antigens was noted with all the monoclonal antibodies, however, the 33 500 MR band obtained with the monoclonal Ly-6.2-specific antibody is clearly specific. A similar unique band was derived from all other Ly-6.2 positive tumors labeled with ^{35}S, but not from Ly-6.2 negative cell lines (see Fig. 2b).

Two dimensional gels showed that Ly-6.2 is basic; four forms of equal size but differing in charge were resolved (Fig. 6).

Although the cell line EL4 gave the best yield of ^{125}I labeled Ly-6.2, one problem we encountered during these experiments was that the Ly-6.2 and T 30 antigens (the latter was immunoprecipitated as a control) tended to aggregate in 0.5% NP 40 lysates and were, therefore, coprecipitated by monoclonal antibodies against either of the antigens. We were able to separate the two antigens by the addition of 1% deoxycholate and 0.1% SDS to the 0.5% NP 40 lysate; we then found that the apparent molecular weight of the T 30 antigen was 25 000. Based on all our

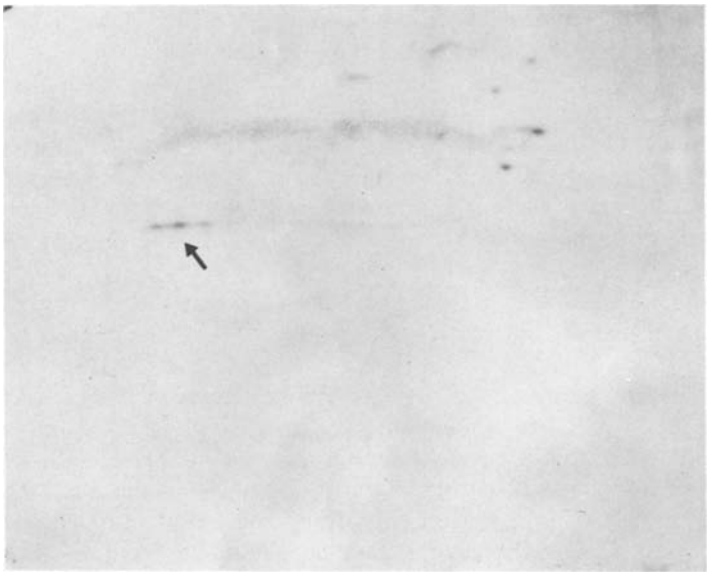

Fig. 6. Two dimensional gel electrophoresis of the Ly-6.2 antigen immunoprecipitated from ^{35}S labeled EL4 tumor cells using anti-Ly-6.2 (S8.106). The first dimension separation was by nonequilibrium pH-gradient electrophoresis (acidic proteins on the right). The second dimension separation (top to bottom) was by SDS-PAGE on 12.5% gels.

observations for the Ly-6.2 antigen, we determined that it has an apparent molecular weight of approximately 33 500.

Discussion

The two protein antigens ThB and Ly-6.2 that we have characterized are clearly different both in terms of apparent molecular weight and charge. ThB was an acidic monomer with an apparent molecular weight of 16 000 and in general was easier to immunoprecipitate than Ly-6.2. The latter was also shown to be monomeric but had a higher apparent molecular weight of 33 500. It was also found that the Ly-6.2 antigen exists in at least four similarly sized forms with different basic charges. These forms presumably reflect differences in post-translational processing involving either glycosylation or amidation.

The difficulty of immunoprecipitation of Ly-6.2 may partly reflect the low antigen density on both normal and activated lymphocytes. We have calculated that the geometric mean density of Ly-6.2 antigen on positive splenic lymphocytes is 9 000 molecules per cell (P. Rogers, A. Matossian-Rogers, and L. A. Herzenberg, unpublished observations). This is approximately $1/40^{th}$ of the number of Thy-1.2 antigens, one-fourth of the number of Lyt-1 antigens and one-seventh of the number of Lyt-2 antigens found on positive splenic lymphocytes (Ledbetter et al. 1980). The number of Ly-6.2 antigens on splenic lymphocytes increases approximately sixfold on Con-A activation but since the cells are bigger, the increase in density per unit

surface area is less. Even where the antigen density was higher, on Ly-6.2 bearing cell lines, there was no correlation between the Ly-6.2 staining intensity and the amount of ^{125}I labeled immunoprecipitate. Indeed, the EL4 tumor, which yielded the most ^{125}I labeled immunoprecipitate, was the least brightly staining with monoclonal Ly-6.2-specific antibody (Table 1). If we assume that the charge differences mentioned earlier are not due to differences in primary structure, then the number of tyrosine residues available for iodination per molecule must vary between cell lines. Hence, there must be differences in the presentation of the Ly-6.2 antigen on different cell surfaces.

Possibly these differences were of less consequence when the cells were metabolically labeled so that the different cell lines yielded quantitatively similar amounts of ^{35}S labeled antigen.

Variation in antigenic presentation may also explain the heterogeneity of Ly-6.2 staining profile seen on the EL4 tumor line and the increase of Ly-6.2 staining intensity on mitogen stimulation of normal lymphocytes.

These experiments also showed that on solubilization of the membrane with 0.5% NP 40, the Ly-6 and T 30 antigens were coprecipitated by either monoclonal Ly-6.2-specific or T 30-specific antibodies. As these antibodies are from different species, and anti-ThB, of the same subclass as anti-T 30, did not immunoprecipitate either antigen, it is most likely that the association is between the two antigens rather than between antigen and antibody. The two antigens were separated by addition of 0.1% SDS and 1% deoxycholate to the lysis solvent. Whether this association is of biological significance we do not know. However, as a consequence of this work, we found that the T 30 antigen expressed on EL4 has an apparent molecular weight on one dimensional electrophoresis of approximately 25K. This is lower than that previously reported (Ledbetter and Herzenberg 1979); the discrepancy might be accounted for by the different sources of the antigen or alternatively might be a consequence of the different gel systems used in the analyses.

Recently Andersson and co-workers (1981) reported an apparent molecular weight of 78 000 for the Ly-6.2 antigen based on immunoprecipitation studies using conventional anti-Ly-6.2 sera and the EL4 tumor. Our results with monoclonal antibodies clearly challenge their findings. The authors were aware of the possibility of contaminating antibodies in their antisera; the use of a monoclonal reagent eliminates this problem.

One important distinction between the two genetically linked antigens, ThB and Ly-6.2, that we have immunoprecipitated is that whereas Ly-6 is known to exist in allelic forms, ThB is not. Rather, the polymorphism associated with ThB is that of low or high expression (Eckhardt and Herzenberg 1980). The regulatory gene is either linked or has identity with genes that code for the lymphocyte antigens Ly-6 and H 9/25 (and possibly Ly-8, Ala-1, and Dag).

To date there is no available information as to the size and nature of these other linked antigens. To obtain such data it will probably be necessary to use tumor cell lines since the density and presentation on normal cells preclude analysis of the antigens by the techniques that are currently available. It is possible of course that antigens characterized from tumor cells are different to those of normal lymphocytes. However, for ThB at least, Con-A and LPS blast cells yield molecules with the

same molecular weights as those found on the tumors; thus, this antigen seems to be the same on both tumors and normal lymphocytes.

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