Monoclonal Antibodies against a Specific Nonhistone Chromosomal Protein of *Drosophila* Associated with Active Genes

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Abstract Hybridomas secreting monoclonal antibodies have been produced by fusion of NS-1 mouse myeloma cells with the spleen cells of mice inoculated with a 60–65,000-mol wt fraction of proteins released from *Drosophila* embryo nuclei treated with DNase I. The antibodies secreted by the hybridomas were examined with polytene chromosomes of formaldehyde-fixed salivary gland squashes by an immunofluorescence assay. Most of the clonal antibodies obtained resulted in specific staining of the chromosomes relative to the cytoplasmic debris. In the case of clone 28, the antibodies showed a preferential association with sites of gene activity, both puffs and loci identified as puffing at some time during the third instar and prepupal period. In larvae that were heat shocked (exposed to 35°C for 15 min before removal and fixation of the glands), the antibodies of clone 28 stained preferentially the induced heat-shock loci while continuing to stain most of the normal set of loci. The antigen for clone 28 was identified as a single protein of ~62,000 mol wt by using the antibodies followed by 121I-rabbit anti-mouse Ig to stain nitrocellulose replicas of SDS polyacrylamide gels of total chromosomal proteins. This study demonstrates that monoclonal antibodies can be used successfully in immunofluorescence staining of formaldehyde-fixed polytene chromosomes. The results verify the hypothesis that a specific nonhistone chromosomal protein is preferentially associated with the set of loci that includes both active sites and those scheduled to be active at some time in this developmental program. Such proteins may play a general role in the mechanisms of cell determination and gene activation.

During the last several years, methods have been developed to study the distribution of chromosomal proteins on the polytene chromosomes of *Drosophila* salivary glands by immunofluorescence techniques (1–4). With the use of such an approach, it has been observed that enzymes involved in gene transcription (such as RNA polymerase) and RNA packaging proteins are found preferentially associated with the active loci (puffs) (5–7). In contrast, antibodies prepared against certain subfractions of the nonhistone chromosomal proteins (NHC proteins) give a staining pattern indicating that some such proteins are preferentially associated with both the active loci and those scheduled to be active (puff) at some time during the third instar larval and prepupal stages (8, 9). Such proteins may play an important role in chromatin structure related to the active state. Unfortunately, it is generally difficult to purify the NHC proteins of interest, as their specific role is unknown and no direct assay is available. With the advent of the monoclonal antibody technology, it is now possible to obtain antibodies specific for a given protein without having available the purified protein as the immunogen. Consequently, one should be able to obtain antibodies specific to the desired NHC proteins, using the immunofluorescence chromosome staining assay as the point of reference, and subsequently to purify these proteins using the antibodies as the point of reference.

A technical problem may arise, as the sensitivity of the immunofluorescence staining procedure with polytene chromosomes is unknown. The chromosomes are fixed with formaldehyde before squashing and spreading, and it is difficult to estimate the percent of antigenic determinants accessible. A direct test is required to demonstrate that an antibody prepa-
ration reacting with a single site per (accessible) antigen is an adequate reagent for this procedure.

We have initiated such work by obtaining monoclonal anti-

bodies against the Band 2 immunogen and using these to stain the polytene chromosomes. Band 2 is a subfraction of the *Drosophila* NHC proteins released following DNase I digestion.

**FIGURE 1** Light micrographs of polytene chromosomes stained with antibodies secreted by clone 28. With this antibody, the staining pattern obtained on chromosomes from animals grown at 25°C indicates a marked preference of the antibody for puffs and for a set of loci identified as puffing at some time during the third instar and prepupal stages (a and b). When chromosomes from animals "heat shocked" at 35°C for 15 min were used in the staining experiments, strong fluorescence is seen at the heat-shock loci with continuing fluorescence at most of the developmentally active loci (c and d). a and c, Phase contrast; b and d, fluorescence micrographs. X 700.
of embryo nuclei (9). DNase I is known to preferentially degrade active loci, releasing RNA polymerase and other chromosomal proteins (10, 11). An antiserum obtained using the Band 2 subfraction as inoculum shows a distribution pattern of prominent association with both the active loci and those known to be active at some time in the polytene chromosomes of the salivary gland (9). We will refer to this set of loci as the developmentally active loci. Such a distribution pattern had also been observed using an antiserum against a subfraction of NHC proteins of 80–110,000 mol wt (8). The Band 2 subfraction contains several polypeptides of 60,000–65,000 mol wt; the antiserum obtained contains primarily antibodies reacting with two of these. The experiments described below demonstrate that such a pattern of association with the developmentally active loci can be obtained for a particular NHC protein of the Band 2 subfraction using monoclonal antibodies.

MATERIALS AND METHODS

Preparation of Immunogen

The immunogen was prepared by the procedure of Mayfield et al. (9). Briefly, nuclei were isolated from frozen 12–18 h Drosophila melanogaster embryos using the method of Hewish and Burgoyne (12). After isolation, the nuclei were exposed to a limited DNase I digestion (to 10–15% acid solubility of the DNA). Proteins released by this treatment were obtained by extracting the nuclei with 0.2 mM EDTA and separated on a 10% SDS polyacrylamide gel (13). The gel band containing the 60–65,000-mol wt subfraction (Band 2 of Mayfield et al. (9)) was excised, dried, and ground to a powder.

Preparation of Antibodies

The gel band powder was suspended in 1 vol of phosphate-buffered saline (0.01 M sodium phosphate, pH 7.6, 0.15 M NaCl), emulsified with 1 vol of Freund’s complete adjuvant, and injected intraperitoneally into BALB/c female mice. A booster injection (identical to the initial injection except that Freund’s incomplete adjuvant was used) was given 30 d after the initial injection. Each
injection contained ~25 μg of total proteins. The procedure used for the cell fusion was that of Ghefer et al. (14) as modified by Shimefield et al. (15). On the 3rd d after the booster injection, the mouse spleen was removed and the spleen cells were dispersed and fused to cells of the NS-1 mouse myeloma line (a MOPC 21-derived cell line carrying hygromycin, ampicillin, thymidine HAT markers obtained from C. Milstein, Cambridge University) at a ratio of 10:1 (spleen cells: NS-1 cells). Hybrid cells were cloned immediately by limiting dilution and selected in HAT medium. With few exceptions, the antibody concentrations of the cultured cell supernates were insufficient for use in the immunofluorescence chromosome staining assay. For this reason, the cell lines were injected intraperitoneally into pristane-primed BALB/c mice and grown as ascites tumors. The resulting ascites fluid was used as the primary source of antibody. Most of the antibodies used in this study were of the IgM type. It was found to be necessary to use fresh ascites fluid for the immunofluorescent chromosome staining experiments. Stored material gave uneven staining, apparently because of problems of IgM aggregation.

Immunofluorescence Assay

The distribution patterns of the antibodies on polytene chromosomes from squash preparations of formaldehyde-fixed D. melanogaster salivary glands were obtained and analyzed by the methods of Silver and Elgin (1, 2, 8, 16). A very high percent of the hybridomas obtained secrested an IgM. In such cases, it is necessary to use a third antibody in the immunofluorescence procedure. The sequence of antibodies used in IgM staining was (a) the monoclonal antibody, (b) rabbit anti-mouse IgM (heavy chain specific), and (c) fluorescein conjugated goat anti-rabbit IgG (Miles Laboratories, Inc., Elkhart, Ind.). Squash preparations of chromosomes from heat-shocked larvae were obtained by incubating larvae at 35°C for 15 min immediately before removal and fixation of the salivary glands. All preparations were examined by phase contrast and fluorescence microscopy.

Preparation of Nitrocellulose Replicas of Polycrylamide Gels

Total D. melanogaster chromosomal proteins were obtained as follows. Isolated chromatin, from nuclei lysed in 0.01 M Tris, pH 8.0 (9), was dissociated with 2.0% SDS, 0.01 M Tris, pH 8.0, and the DNA was removed by centrifugation. The chromosomal proteins were separated on a 10.0% SDS polyacrylamide gel (13) and transferred electrophoretically to nitrocellulose (17). Incubation of the nitrocellulose replica with 3% bovine serum albumin (BSA), 0.35 M NaCl, 0.01 M Tris, pH 7.5, 0.1 mM phenylmethylosulfonyl fluoride (PMSF) eliminated the remaining protein binding sites. The antigen band was visualized by incubation with the monoclonal antibody (diluted 1:200 or 1:1,000 in 3% BSA, 0.5 mg/ml bovine gamma globulins, 0.01 M Tris, pH 7.5, 0.35 M NaCl, 0.1 mM PMSF) for 2-6 h at room temperature followed by washes with 0.35 M NaCl, 0.01 M Tris, pH 7.5, followed by incubation with 125I-rabbit anti-mouse Ig (105 cpm/ml) for 2-6 h at room temperature in the same incubation solution as above, further washes, drying, and autoradiography. Additional samples of the total chromosomal proteins of protein molecular weight standards, run on the same gel and transferred simultaneously to nitrocellulose, were stained with amido black. The mobilities of the standards were measured directly from the nitrocellulose, and the molecular weight of the gel band stained by clone 28 antibodies was determined by the use of a plot of mobility versus log molecular weight of the standards.

RESULTS

The Band 2 immunogen of Mayfield et al. (9) was used to inoculate BALB/c female mice. Antibodies from hybridoma cell lines obtained from the fusion of spleen cells from the immunized mice with cells of the NS-1 mouse myeloma cell line were examined for their distribution pattern on polytene chromosomes by use of an immunofluorescence assay (1, 2, 8, 16). While staining the chromosomes specifically, relative to the cytoplasmic debris, the antibodies from many of the clones examined showed a more general distribution, staining a larger set of loci, than had the Band 2 antisera. The patterns were, however, distinct and may be investigated in detail in the future. In the case of clone 28, the distribution pattern obtained showed that the antibodies had a marked preference for the active regions of the chromosomes (the puffs) and a relatively small subset of additional loci (Fig. 1, a, and b). Upon careful examination, this set of loci can be correlated closely with the set of loci described by Ashburner (18) as being active (puffed) at some time during the third instar or prepupal developmental stages in the salivary gland (the developmentally active loci). Of 51 loci on chromosome arms 3R and 3L classified as staining the clone 28 antibodies, 44 are known to form major puffs at some stage (Table I). This staining of sites that are presently active and that have been or will be active during the third instar and prepupal stages is very similar to that obtained by Silver and Elgin (8) and by Mayfield et al. (9) for the ρ and Band 2 antisera, respectively. The former results are included in Table I for comparison.

To further examine the association of the clone 28 antibody with active regions of the genome, we prepared salivary gland squashes from heat-shocked larvae at 35°C for 15 min immediately before removal and fixation of the glands. D. melanogaster exposed to this higher temperature exhibit a heat-shock response in which genes normally active are turned off and a new set of nine heat-shock loci are activated (19). Staining of formaldehyde-fixed chromosome preparations from heat-shocked animals with clone 28 antibodies showed prominent fluorescence at all the heat-shock puffs (e.g., 63BC, 67B, 70A, 87A, 87BC-Cl, 93D, and 95D) with continuing fluorescence at most of the normally active developmental loci (e.g., 13E and 50C) (Fig. 1e and f). Staining is lost or reduced at ~20% of the developmentally active loci (e.g., 2B and 85F).

As it had done in the case of the chromosomes from larvae grown at 25°C, the clone 28 antibody stained chromosomes of heat-shocked larvae at the immediately active (heat-shock) loci and at most of those other loci which had been or would be active (Table I). The results are generally similar to those obtained with the ρ antisera and the Band 2 antisera. The most striking difference is the loss of staining in the clone 28 case at ~20% of the prominent developmental loci (e.g., 85F). A direct comparison between the clone 28 distribution pattern and that of the ρ and the Band 2 antisera can be seen in Fig. 2. In this figure, the staining of chromosome arm 3R from heat-shocked larvae is compared.

To identify the specific antigen reacting with clone 28 antibody, we separated total chromosomal proteins on a 10% SDS polyacrylamide gel and transferred them to nitrocellulose (17). When the nitrocellulose gel replica was incubated with the clone 28 antibodies and subsequently with 125I-rabbit anti-mouse Ig, autoradiography indicated binding of the antibody to a single protein band of ~62,000 mol wt (Fig. 3). The signal-to-noise ratio obtained with clone 28 antibodies in this type of assay was not so great as routinely obtained with most antisera; however, identical results were obtained in four independent experiments, giving confidence in the observations.

DISCUSSION

The results obtained demonstrate that a particular NHC protein of ~62,000 daltons is preferentially associated with active loci (puffs) and sites that will be or have been active during the third instar and prepupal periods in the salivary gland. Despite the necessity for chromosome fixation with formaldehyde, the sensitivity of the immunofluorescence staining method on polytene chromosomes is adequate to obtain good results with monoclonal antibodies, specific for a single determinant. These observations further indicate the usefulness of this cytological approach to the study of chromatin structure. The specific staining pattern seen here suggests that this NHC protein of ~62,000 daltons is one (perhaps part of a set of proteins) whose
presence is necessary but not sufficient for gene transcription. This statement is, of course, based on a correlation that is good but not perfect (see Table I). Given inherent limitations of cytological analysis, it seems likely that biochemical approaches will be needed to test this proposition further. The monospecificity of the clone 28 antibody confirms that this distribution pattern is characteristic of a particular, single protein. The antibody should be useful in isolating this NHC protein for further study.

The clone 28 antibody and the Band 2 antiserum were made in response to the same protein fraction of 60,000–65,000 mol wt. Although very similar, the distribution patterns obtained with these two antibody preparations are not identical. This may be partially explained by the polyspecificity of the Band 2 antiserum. In experiments analogous to those described above, staining of two prominent protein bands by Band 2 antiserum in a nitrocellulose gel replica of total chromosomal proteins is observed (data not shown). Based on molecular weight measurements, the clone 28 antigen is apparently the smaller of these two. It is also possible that in some cases the clone 28 antigen is present in a form such that it is not recognized by the monospecific reagent, but is recognized by the Band 2 antiserum. Such questions can only be resolved by obtaining the purified protein for use in appropriate experiments.

The pattern of association of the clone 28 antigen with the polytene chromosomes suggests that this protein might be a component of the chromatin structure correlated with the general DNase I sensitivity of active loci. Such a configuration appears to be necessary but not sufficient for gene activity (see references 20 and 21 for a review). This suggestion is supported by a comparison of the observations on the developmentally active loci and on the heat-shock loci. Genes active in terminally differentiated cells have been reported to be generally sensitive to DNase I, regardless of whether or not they are actually being transcribed at the moment of assay (globin, 11; ovalbumin, 22). In contrast, the heat-shock loci, which can be induced in any Drosophila cell, show a relative shift in DNase I sensitivity correlated with the induction of transcription (23). The developmentally active loci in the salivary gland, a terminally differentiated cell, are continuously associated with the clone 28 antigen, before, during, and after puffing. In contrast,

### Table I

Comparison of Bands Stained by Clone 28 Antibodies and the ρ Antiserum to Those Identified by Ashburner (18) as Puffing during Third Instar

| Locus  | Puffed, room temp | Puffed, heat shock | ρ Staining, room temp | ρ Staining, heat shock | Clone 28 staining, room temp | Clone 28 staining, heat shock | Clone 28 staining, room temp | Clone 28 staining, heat shock |
|--------|------------------|-------------------|-----------------------|-----------------------|-----------------------------|-------------------------------|-----------------------------|-------------------------------|
| 61A    | −                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 61C    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 62A    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 62E    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 63B    | −                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 63E    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 63F    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 64AB   | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 64F    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 66B    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 66E    | +                | −                 | −                     | −                     | +                           | +                             | +                           | +                             |
| 67B    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 67F    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 68C    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 69A    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 70A    | −                | +                 | −                     | −                     | +                           | +                             | +                           | +                             |
| 70C    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 71B    | +                | −                 | −                     | −                     | +                           | +                             | +                           | +                             |
| 71DE   | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 72D    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 73B    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 74C    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 74EF   | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 75B    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 76A    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 76CD   | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 77E    | +                | −                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 78C    | −                | +                 | −                     | −                     | +                           | +                             | +                           | +                             |
| 78D    | +                | +                 | +                     | +                     | +                           | +                             | +                           | +                             |
| 79EF   | +                | −                 | −                     | −                     | +                           | +                             | +                           | +                             |

* Loci identified as puffing during 3-d instar or prepupal development according to Tables IV and V of Ashburner (18).
† Heat-shock loci—loci identified as puffing in response to an increase in temperature according to Ashburner (18).
‡ Staining pattern as published by Silver and Elgin (8).
\(\dagger\) Parentheses indicates a less intense or inconsistent staining.
** Major puff observed by Zhimulev (28), but not by Ashburner (18).
the heat-shock loci, such as 87A and 87B-C1, are prominently stained with clone 28 antibodies only when they have been induced to puff.

Other nonhistone chromosomal proteins thought to be associated with active loci are the high mobility group (HMG) proteins, a set of small, lysine-rich proteins. Weisbrod et al. (24, 25) have demonstrated by extraction and reassociation techniques that the HMG proteins 14 and 17 are found in association with DNase I-sensitive chromatin in chick erythrocytes and are necessary to maintain the DNase I-sensitive state. This work and the work of others has stimulated a great interest in the HMG proteins. However, the clone 28 antigen does not
This work was supported by the National Science Foundation grant PCM-78-23709 awarded to S. Elgin. G. Howard is a Fellow of the Damon Runyon-Walter Winchell Cancer Fund. V. Sato is supported by Research Career Development Award CA00512 and S. Elgin is supported by Research Career Development Award GM00234.

Received for publication 20 August 1980, and in revised form 9 October 1980.

Note Added in Proof: Staining of Drosophila polytene chromosomes with monoclonal antibodies against chromosomal proteins has also been reported by H. Sauumweber, P. Symmons, R. Kabas, H. Will, and F. Bonhoeffer, 1980. Monoclonal antibodies against chromosomal proteins of Drosophila melanogaster. Chromosoma (Berl), 80:253-288.

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Figure 3 Comparison of chromosomal proteins separated on a 10% SDS polyacrylamide gel and stained with Coomassie Blue (a) with a nitrocellulose replica of the same gel “stained” with antibodies from clone 28 followed by 125I-rabbit anti-mouse Ig visualized by autoradiography (b). The antibodies bind to a protein band of ~62,000 molecular weight.

The 125I-rabbit anti-mouse Ig was generously provided by Larry J. Wysocki.

1 Serunian, L., and S. C. R. Elgin. Unpublished results.