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On the nature of crossreactions observed with antibodies directed to defined epitopes

(anti-peptide antibodies/monoclonal antibodies/immunofluorescence)

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ABSTRACT **Antibodies directed against a synthetic peptide (*src*-c) containing the six carboxyl-terminal amino acids of $p60^{src}$, the transforming protein of Rous sarcoma virus, recognize $p60^{src}$. However, when used at sufficiently high concentrations they also react with a number of constituents of untransformed cells. These reactions can be completely inhibited by *src*-c peptide. Crossreactivities are to different components in cells from different species and cannot be attributed to $p60^{c\text{-}src}$, the ubiquitous cellular homologue of $p60^{src}$. By indirect immunofluorescence microscopy and immunochemical techniques we have identified three cytoskeletal proteins, myosin, tubulin, and vimentin, as well as an unknown intranuclear antigen, as major targets of anti-*src*-c antibodies in different untransformed cells. These crossreactivities probably reflect identities or similarities in the amino acid sequence of the immunogenic peptide and segments of the otherwise unrelated crossreactive proteins. These findings are discussed with respect to the interpretation of crossreactivities that are occasionally observed with anti-peptide sera and with monoclonal antibodies.**

An attractive possibility for producing antisera against particular proteins is to immunize animals with hapten conjugates made with synthetic oligopeptides corresponding in sequence to selected portions of these proteins (1–8). Because rapid progress in nucleotide sequence analysis allows the prediction of amino acid sequences of an increasing number of proteins, it may be expected that this synthetic approach to the production of monospecific antibodies will find widespread applications in the near future.

Recently antibodies have been raised to a synthetic peptide (*src*-c) corresponding to the carboxyl-terminal hexapeptide sequence of $p60^{src}$, the transforming protein kinase coded for by the *src* gene of Rous sarcoma virus (RSV). The amino acid sequence was deduced from the nucleotide sequence (9). These antibodies (referred to as anti-*src*-c) were then used to investigate the intracellular distribution of $p60^{src}$ (10). By using low concentrations of affinity-purified anti-*src*-c antibodies, specific immunofluorescent labeling of $p60^{src}$ could be achieved. However, with increasing concentrations of antibody we observed labeling of constituents present in untransformed cells or in cells transformed by agents other than RSV. In the present study, these crossreactivities were investigated in detail in order to determine their significance, and in particular whether they might reveal functional or structural relationships between $p60^{src}$ and the crossreactive normal cell constituents. The results of this study, however, have suggested to us that these crossreactions are fortuitous, but they may occur with some frequency with such anti-peptide antisera. These results also bear on the interpretation of unexpected crossreactivities observed occasionally with monoclonal antibodies.

MATERIALS AND METHODS

Cell Culture and Immunochemical Reagents. Untransformed and RSV strain B77-transformed normal rat kidney cells (NRK and NB77 cells, respectively) were cultured as described (10) from stocks originally provided by Peter K. Vogt (University of Southern California, Los Angeles). Mouse fibroblasts [BALB 3T3 and NIH Swiss 3T3 transformed by simian virus 40 (SV40)] were from B. M. Sefton (The Salk Institute, San Diego, CA) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Baby hamster kidney cells (BHK-21) were grown in Joklik's minimal essential medium supplemented with nonessential amino acids and 4% fetal calf serum. Chicken embryo fibroblasts were cultured as described (11).

The synthesis of the heptapeptide Tyr-Val-Leu-Glu-Val-Ala-Glu, the production of anti-*src*-c antisera, and the affinity purification of peptide-specific antibodies have been reported in detail (8, 10). Antibodies to avian brain tubulin (12) as well as fluorescein and rhodamine conjugates of goat anti-rabbit IgG (11) have also been described.

Indirect Immunofluorescence Microscopy and Immunochemical Techniques. Cells were cultured on 18-mm^2 coverslips for at least 48 hr prior to immunofluorescence experiments. Indirect immunofluorescent labeling and microscopy were carried out as described (10). For immunochemical characterization of antibody specificity, whole cell extracts were electrophoresed on $NaDodSO_4$/polyacrylamide gels. Blotting of the gels onto nitrocellulose paper was carried out according to Towbin *et al.* (13), using radioiodinated staphylococcal protein A for detection of antigen–antibody complexes.

RESULTS

Immunofluorescent Labeling of Cultured Fibroblasts by Anti-*src*-c Antibodies. Fig. 1 summarizes immunofluorescent staining patterns produced in cultured fibroblasts by either low (*A* and *B*) or high (*C–I*) concentrations of anti-*src*-c antibodies. When used at low concentrations (1–3 μg/ml), these antibodies produced a characteristic staining of RSV-transformed rat cells (Fig. 1*A*), whereas no significant labeling of untransformed rat cells was detectable (Fig. 1*B*). As discussed in detail elsewhere (10), the staining pattern of RSV-transformed fibroblasts clearly reflects the intracellular distribution of $p60^{src}$. At higher concentrations of antibody (10–20 μg/ml), however, a punctate

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Abbreviations: RSV, Rous sarcoma virus; SV40, simian virus 40.

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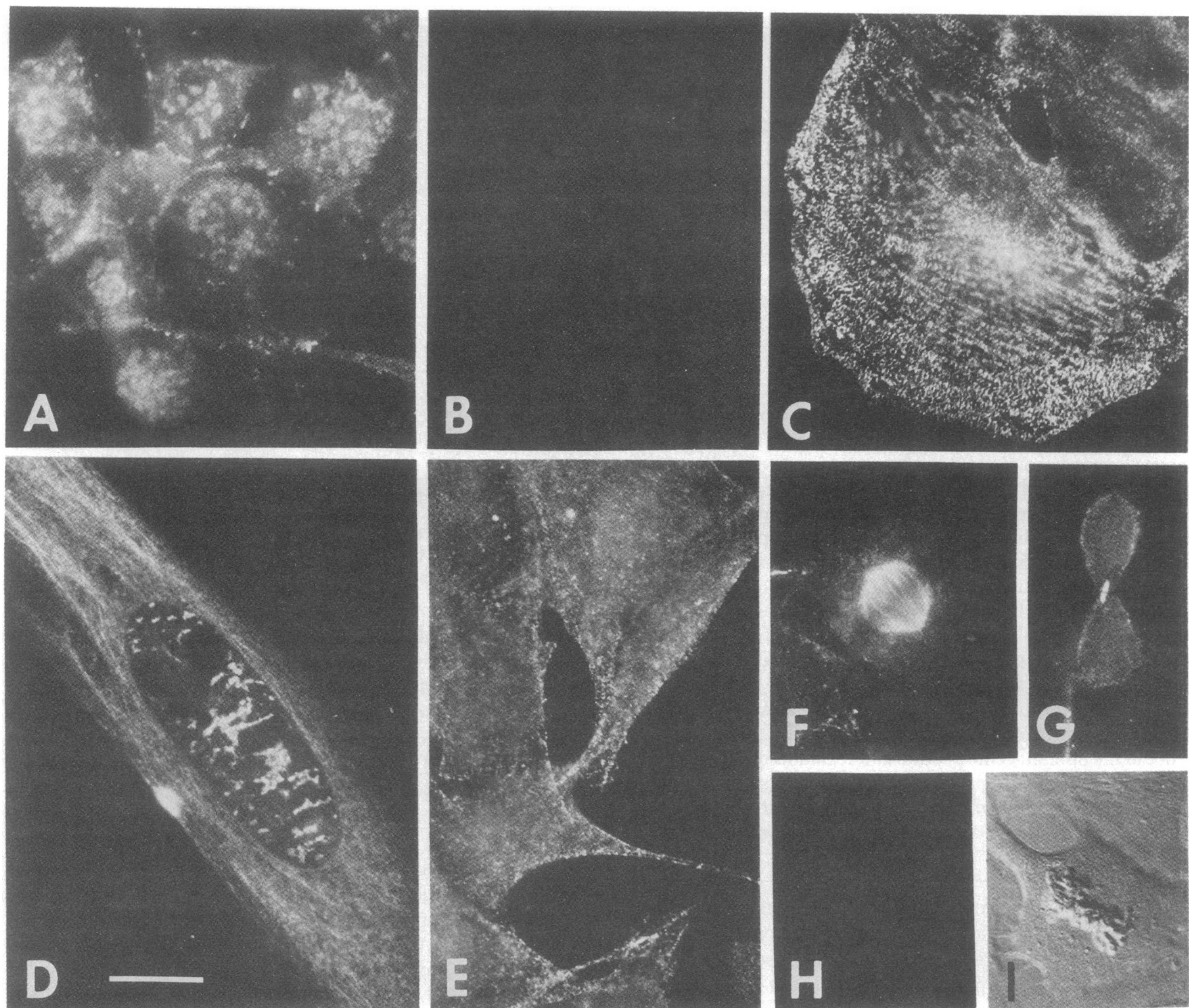

FIG. 1. Indirect immunofluorescent staining patterns produced by affinity-purified anti-*src*-c antibodies in cultured fibroblasts. Fixed and permeabilized cells were incubated with rabbit anti-*src*-c IgG at 2 μg/ml (*A* and *B*) or 15 μg/ml (*C*–*I*) and subsequently with fluorescein- (*A* and *B*) or rhodamine- (*C*–*I*) conjugated goat anti-rabbit IgG. (*A*) NB77 cells; (*B* and *C*) NRK cells; (*D*) chicken embryo fibroblasts; (*E*) SV40-transformed mouse fibroblasts; (*F*–*I*) NRK cells in metaphase (*F*, *H*, and *I*) and after completion of mitosis (*G*). Prior to staining of the metaphase cell in *H* and *I*, affinity-purified anti-*src*-c IgG was incubated for 10 min at 4°C with soluble *src*-c peptide at 200 μg/ml. *I* shows the same cell as in *H* when viewed by Nomarski optics. Bar in *D* represents 20 μm.

and often fibrillar staining pattern became apparent in untransformed NRK cells (Fig. 1*C*). This pattern resembles the distribution of myosin in stress fibers. Very similar patterns were obtained in rat, mouse, and chicken fibroblasts. In chicken cells, however, an additional prominent intranuclear fluorescence was consistently observed, along with a wavy fibrillar cytoplasmic staining that was occasionally seen (Fig. 1*D*). The wavy pattern was similar to that observed in the specific immunofluorescent labeling of microtubules or intermediate filaments in these cells. Transformation of fibroblasts by a variety of agents other than RSV (i.e., the DNA virus SV40 shown in Fig. 1*E*) resulted in diffuse cytoplasmic staining by anti-*src*-c antibodies. Furthermore, rat, mouse, or chicken cells in mitosis or shortly after mitosis showed very prominent fluorescent labeling of the mitotic spindle (Fig. 1*F*) and the cytoplasm immediately adjacent to the midbody in postmitotic bridges (Fig. 1*G*), respectively, closely resembling the distribution of tubulin in these structures. We emphasize that all staining patterns shown in Fig. 1 are specific in that they can be completely inhibited by an excess of soluble *src*-c peptide (see, for example, Fig. 1 *H* and *I*). Virtually identical results were obtained by using anti-*src*-c antibodies prepared from different rabbits, and no labeling was produced by antibodies contained in the flow-through of the affinity column used to purify anti-*src*-c specific IgG (not shown).

Biochemical Identification of Immunoreactive Proteins. For immunochemical analysis of antibody specificity (Fig. 2), whole cell extracts were resolved on $NaDodSO_4$/polyacrylamide gels and transferred to nitrocellulose paper. Paper strips were either stained with amido black (lanes A and B) or sequentially incubated with anti-*src*-c antibodies and radioiodinated protein A. Autoradiography revealed only one major band, corresponding to $p60^{src}$ present in RSV-transformed (lane C) but not in untransformed NRK cells (lane D), when low concentrations of anti-*src*-c antibody were employed. This result is consistent with the specific immunofluorescent staining of $p60^{src}$ achieved by using low concentrations of antibody (10). Labeling of additional proteins in the blotting experiments was

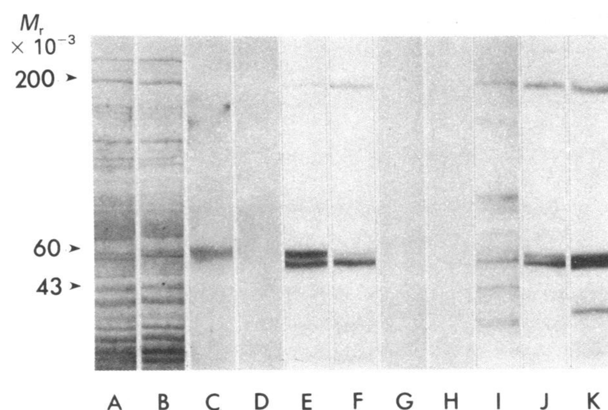

FIG. 2. Whole cell extracts were prepared by solubilizing cultured cells in boiling gel sample buffer. After high-speed centrifugation (100,000 × g, 30 min) extracts were resolved on $NaDodSO_4$/8% polyacrylamide gels and transferred to nitrocellulose paper. Paper strips were either stained with amido black (lanes A and B) or incubated with anti-*src*-c antibodies followed by ^{125}I-labeled protein A at 20,000 cpm/ml and autoradiography (lanes C–K). After application of each layer of reagent, paper strips were washed six times for 5 min each at room temperature. Extracts were from NB77 cells (lanes A, C, E, and G), NRK cells (lanes B, D, F, and H), BHK (lane I), BALB 3T3 (lane J), and chicken embryo fibroblasts (lane K). In lanes C and D, anti-*src*-c IgG was used at 0.12 μg/ml and autoradiography was for 5 days; in lanes E–K, anti-*src*-c IgG was used at 3 μg/ml and autoradiography was for 5 hr. In lanes G and H, in addition to the anti-*src*-c IgG, soluble *src*-c peptide was present at 90 μg/ml during antibody incubation. Arrowheads indicate the positions of molecular weight markers; from top to bottom; myosin heavy chain (200,000), catalase (60,000), and actin (43,000). The lower band in lane E corresponds to molecular weight 55,000.

observed, however, when higher concentrations of antibody were employed (lanes E–K). Immunostaining of these proteins was abolished by the presence of soluble *src*-c peptide during antibody incubations (lanes G and H). Bands of molecular weights of about 200,000, 58,000, and 55,000 were detectable in cell extracts from most species tested, namely rat (lane F), hamster (lane I), mouse (lane J), and chicken (lane K), although the bands were of various intensities. In hamster cell extracts, a few additional faint bands could be visualized (lane I), and in chicken cells one major additional band of molecular weight 35,000–40,000 was observed (lane K). Comparison of these results with the immunofluorescent staining patterns suggested that the molecular weight 200,000 and 55,000 proteins might correspond to the cytoskeletal proteins myosin (heavy chain) and tubulin, respectively. Accordingly, we tested the anti-*src*-c antibodies for reactivity towards individual purified cytoskeletal proteins electrophoresed on $NaDodSO_4$/polyacrylamide gels (results not shown). In blotting experiments these antibodies did react, although weakly, with rat skeletal muscle myosin and, strongly, with avian brain tubulin. A further positive reaction was found with vimentin purified from BHK cells. All these reactions could be completely inhibited by soluble *src*-c peptide. Under the same experimental conditions we did not detect any reactivity of anti-*src*-c antibodies towards purified chicken gizzard vinculin, α-actinin, actin, filamin, tropomyosin, and the microtubule-associated τ proteins. Interestingly, we also found no reaction towards myosins from chicken gizzard and human uterus, indicating that the particular site recognized by anti-*src*-c antibodies on fibroblastic cytoskeletal myosin is absent from smooth muscle myosins or is inaccessible. On the basis of immunochemical evidence and immunofluorescent staining patterns we have thus identified myosin, tubulin, and vimentin from particular cellular sources as quantitatively major proteins crossreacting with anti-*src*-c antibodies. The intranuclear antigen detected by immunofluorescence in chicken fibroblasts was not further characterized, but it might possibly correspond to the low molecular weight protein that was immunoreactive in chicken cell extracts (Fig. 2, lane K).

Homogeneity of Antibody Specificity. The possibility was considered that the anti-*src*-c antibodies are a mixture of antibodies with different specificities either towards different portions or towards different conformations of the carboxyl-terminal hexapeptide, and the apparent crossreactivities between p60^{src} and the normal cell proteins were due to reactions of these different antibodies. To examine this possibility, affinity-purified anti-*src*-c antibodies were passed over an affinity column prepared with avian brain tubulin, one of the positively identified normal cell antigens. After low-pH elution of the specifically retained antibodies, the eluted fraction as well as the column flow-through were tested for their ability to stain untransformed and B77-transformed NRK cells (Fig. 3). Consistent with the retention of anti-tubulin activity on the column, the flow-through fraction had lost its ability to stain postmitotic bridges (Fig. 3 *A* and *B*, arrowheads) and mitotic spindles (Fig. 3 *A* and *B*, *Insets*), but, simultaneously, myosin-like staining of interphase NRK cells (Fig. 3 *A* and *B*) and staining of p60^{src} in RSV-transformed cells (Fig. 3*C*) were also largely abolished. Conversely, antibodies eluted from the tubulin column not only stained mitotic spindles (Fig. 3*D Inset*) and postmitotic bridges (Fig. 3*D*, arrowhead) in untransformed cells but also restored the p60^{src} specific staining pattern (10, 15) in B77-transformed cells (Fig. 3*E*). When used at higher concentrations, these antibodies also produced the myosin-like staining of interphase untransformed cells (not shown). As a control, Fig. 3*F* shows that bona fide anti-tubulin antibodies *per se* do not stain in a pattern coincident with p60^{src} in B77-transformed NRK cells. Finally, when anti-*src*-c antibodies were passed over affinity columns containing chicken gizzard myosin, α-actinin, or tropomyosin—i.e., proteins that were not recognized by the anti-*src*-c antibodies—the flow-through did not lose the capability of staining either normal or transformed cells (not shown). These experiments thus prove that anti-*src*-c antibodies, despite the possibility of their polyclonal origin, display very restricted specificity, and their reactivity towards normal cell proteins reflects a true crossreactivity with homologous or closely similar epitopes on these protein molecules and the p60^{src} carboxyl terminus.

DISCUSSION

We have demonstrated that antibodies directed to a synthetic *src*-peptide when used at very low concentrations react specifically with p60^{src} in RSV-transformed cells (10). However, when used at higher concentration, these antibodies crossreact detectably with constituents of untransformed cells. The crossreactions are specific, because they can be inhibited by an excess of the soluble *src*-c peptide, and because absorption of the anti-*src*-c antibodies with a crossreacting protein (e.g., tubulin) removes the reactivity to p60^{src}. Because the amount of p60^{src} present in transformed cells is 1/100th to 1/1,000th as much as the amount of such abundant proteins as myosin and tubulin, and p60^{src} is detectable at about 1/10th to 1/5th the antibody concentrations, it may be concluded that p60^{src} is recognized by the anti-*src*-c antibodies with about 10^3–10^4 times higher affinity than myosin or tubulin. However, as judged from immunofluorescence microscopy, anti-*src*-c antibodies have comparable affinities for p60^{src} and the antigen present in chicken cell nuclei, thus indicating that crossreactive antigens may not necessarily be bound with widely different affinities.

With regard to the biological significance of the crossreac-

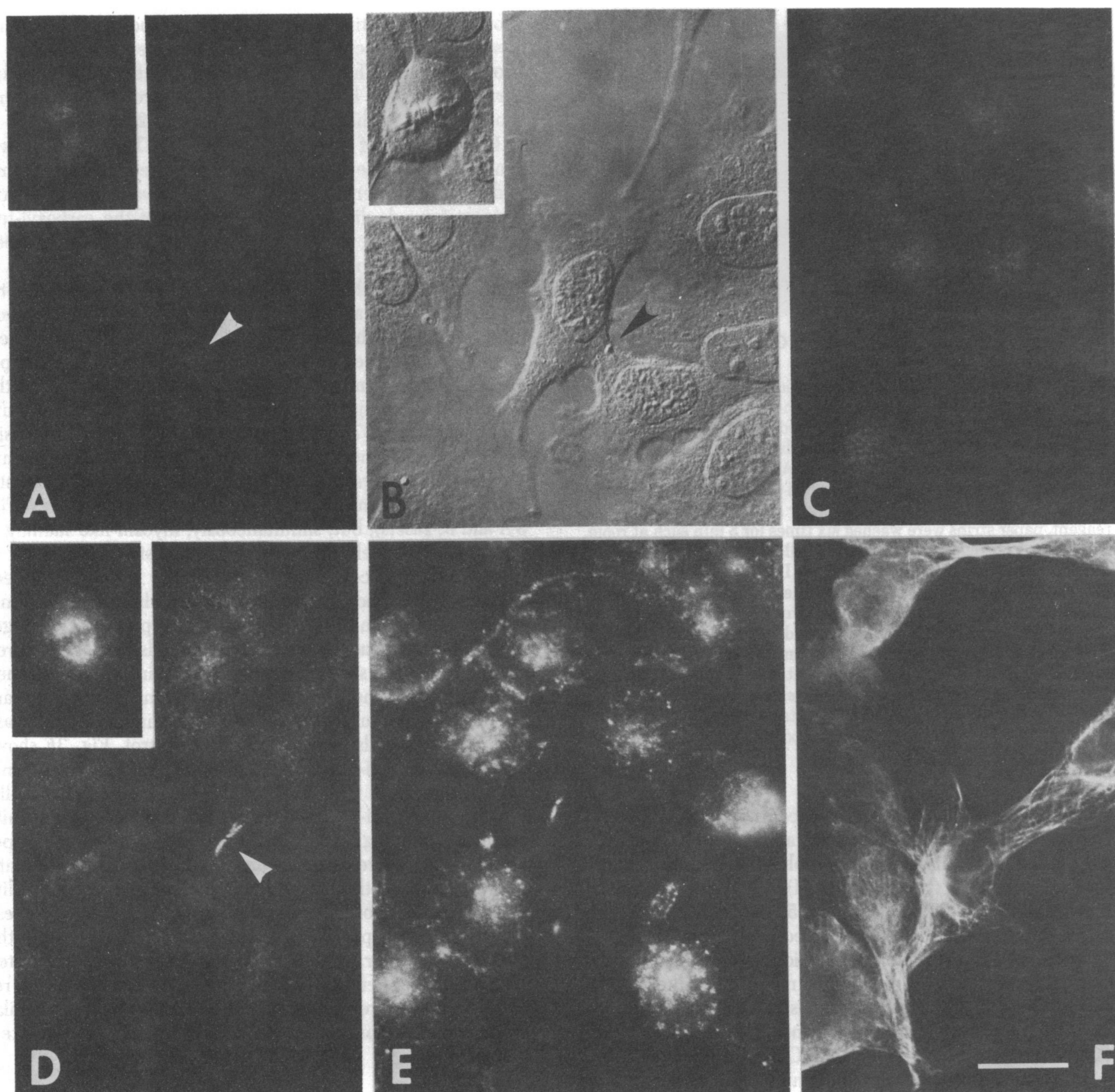

FIG. 3. Affinity-purified anti-*src*-c IgG (100 μg in 0.8 ml) was incubated with avian tubulin coupled to glutaraldehyde-activated Ultrogel AcA 22 (LKB) according to Ternynck and Avrameas (14) in 2.5 ml of gel slurry. After gentle agitation overnight at 4°C the gel slurry was packed into a column and the flow-through was collected. After extensive washing of the column with phosphate-buffered saline, retained antibodies were eluted with 0.2 M HCl adjusted to pH 2.2 with 2 M glycine, neutralized with 0.25 vol of 1 M K_2HPO_4, and dialyzed overnight against phosphate-buffered saline. Column flow-through was used undiluted for staining of NRK (*A* and *B*) and NB77 cells (*C*); eluted IgG was used at 6 μg/ml for staining of NRK (*D*) and NB77 cells (*E*). Staining of NB77 cells by anti-avian tubulin antibodies (10 μg/ml) is shown in *F*. Secondary antibodies were rhodamine-conjugated goat anti-rabbit IgG. In *C* and *E* the microscope was focused to the level of the substrate to allow visualization of immunolabeled residual focal adhesion plaques and transformation-induced rosette clusters (10, 15). *Insets* show mitotic spindles. See text for further details. Bar in *F* represents 20 μm.

tivities reported here, there is no evidence to suggest that they reflect any functional or evolutionary relationships between $p60^{src}$ and the cellular proteins recognized by anti-*src*-c antibodies. In particular, as shown elsewhere (8), no immunochemical evidence could be obtained for a reaction of these antibodies with $p60^{c\text{-}src}$, the cellular homologue of the viral gene product, suggesting that viral and cellular $p60^{src}$ proteins may differ in their carboxyl termini. Considering that different sets of proteins are recognized in different normal cells, it is very likely that the observed crossreactivities reflect fortuitous similarities in the primary sequences of the immunogenic peptide and segments of the reactive proteins. This notion could be verified in the case of β-tubulin, one of the identified crossreactive proteins. Comparison of the amino acid sequences of $p60^{src}$ [ref. 9; the sequence of the amino-terminal third of the molecule has been revised (16) and avian brain tubulin (17) does not reveal any large-scale similarities. However, β-tubulin contains the sequence Val-Leu-Asp-Val-Val-Arg (residues 116–121), the first four of which are remarkably similar in sequence to residues 2–5 of the *src*-c peptide Tyr-Val-Leu-Glu-Val-Ala-Glu. If the anti-*src*-c antibodies were predominantly directed to this tetrapeptide portion of the sequence, the closely similar se-

quence in β-tubulin would also be recognized by the anti-*src*-c antibody. To the extent that the remaining amino acids were involved in the antigenic determinant, the crossreactivity between the *src*-c peptide and tubulin would be reduced. The complete amino acid sequences of rat skeletal muscle myosin and BHK vimentin are not available for similar comparisons.

The crossreactivities of anti-*src*-c antibodies reported here do not represent an isolated phenomenon with such antibodies. A similar crossreactivity has recently been observed with anti-peptide antibodies directed to a sequence on the polyoma middle-size tumor antigen (2), although the reactive proteins were not definitively identified. Clearly, when designing or interpreting experiments based on the use of anti-peptide antisera, it is important to take into account the possibility of such crossreactions. One factor in the design of such experiments is that the immunoreactivity of the anti-peptide antibodies is likely to be more specific for the protein in question if the peptide that is chosen to be used as a hapten contains some relatively less frequently occurring amino acids and is therefore more likely to have an unusual amino acid sequence. Despite the possible complications that may arise from crossreactions, anti-peptide antibodies undoubtedly provide powerful tools for a wide range of purposes. As shown in detail elsewhere (10), anti-*src*-c antibodies used under appropriate conditions and in conjunction with proper controls allowed the immunofluorescent localization of $p60^{src}$ inside RSV-transformed cells. Moreover, $p60^{src}$ can be purified under very mild conditions by immunoaffinity chromatography on columns containing anti-*src*-c antibodies by using soluble *src*-c peptide for elution of retained proteins (unpublished data). Finally, one may wish to take advantage of crossreactivities by designing peptides for immunizations to detect proteins with related functions that might have regional homologies in primary sequences.

Presumably the immunoglobulins used here are not of strictly monoclonal origin, but they resemble monoclonal antibodies in that they react with a single epitope or at most with a small number of overlapping epitopes. Our results may therefore be relevant to the interpretation of crossreactivities as they are occasionally observed with monoclonal antibodies (i.e., see refs. 18–23). In the case of monoclonal antibodies the chemical nature of the immunogenic site is generally unknown, but in the case of anti-peptide reagents this site is very well defined as a short amino acid sequence, and the interpretation of crossreactivities is considerably facilitated. Crossreactivities observed with monoclonal antibodies may often be reflections of functional or evolutionary relationships between the crossreacting proteins, but it must be appreciated that identical or closely similar epitopes may occasionally occur on otherwise unrelated proteins, as we have suggested is the case for the *src*-c peptide and β-tubulin. For example, the crossreaction observed with an IgM monoclonal antibody between a membrane surface antigen, Thy-1, and an unidentified component of intermediate filaments (21) may well be due to the fortuitous presence of a similar epitope on the two proteins involved, a possibility considered by the investigators. Particularly in cases in which a cytoskeletal constituent is implicated in such crossreactions, the large concentrations of such proteins in the cell may be a factor in the detection of the crossreaction by immunofluorescence experiments. It is by no means an original observation, but it should be more widely recognized that crossreactions found with monoclonal antibodies cannot be taken *a priori* to suggest a significant relationship between the proteins involved. This is appreciated for the case in which the monoclonal antibodies turn out to be directed to a carbohydrate determinant, but is not as well appreciated when the antibody is directed to a polypeptide epitope.

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