Hood, M. A. Raftery, *Biochem. Biophys. Res. Commun.* **91**, 164 (1979).
19. We calculated the percentage of protein with the major sequence for each subunit relative to the total protein applied to the sequenator. This was done by assuming 95 percent recovery of the *P*th aliphatic amino acids (*18*) and extrapolating the observed yields of these *P*th amino acids in the sequenator runs back to cycle 0. By comparing these quantities from the sequenator analysis to those for the total protein obtained by amino acid analyses of separate portions of each subunit, we obtained initial yields of 80 percent (for 9.6 nmole of 40 × 10^3 dalton subunit), 80 percent (for 3.1 nmole of 50 × 10^3 dalton subunit), 80 percent (for 2.9 nmole of 60 × 10^3 dalton subunit), and 91 percent (for 11.0 nmole of 65 × 10^3 dalton subunit).
20. The preparation of the 60 × 10^3 dalton subunit contained some 50 × 10^3 dalton subunit (~15 percent) as a contaminant.
21. J. Lindstrom, B. Walter, B. Einarson, *Biochemistry* **18**, 4470 (1979).
22. T. Claudio and M. A. Raftery, *J. Immunol.* **124**, 1130 (1980).
23. J. Lindstrom, J. Merlie, G. Yogeeswaran, *Biochemistry* **18**, 4465 (1979); S. C. Froehner and S. Rafto, *ibid.*, p. 301; N. Nathanson and Z. Hall, in (*31*).
24. Subunit stoichiometry was calculated by comparing the yields of the *P*th amino acids assigned to positions 9, 10, and 14 of the four subunits where each of the four subunits has a different amino acid residue. The yields of the *P*th amino acids were calculated by subtracting the background *P*th amino acid levels and then correcting for the differing recoveries of the *P*th amino acids (alanine, 95 percent; serine, 35 percent; glutamic acid, 80 percent; asparagine, 65 percent; valine, 95 percent; lysine, 50 percent; aspartic acid, 60 percent; and threonine, 40 percent) observed in sequenator analysis of the isolated subunits. These values represent the efficiency of conversion of the amino acid thiazolinones to the corresponding *P*th amino acids in the sequenator.
25. M. Ross, M. Klymkowsky, D. Agard, R. M. Stroud, *J. Mol. Biol.* **116**, 635 (1977).
26. E. Nickel and L. T. Potter, *Brain Res.* **57**, 508 (1973); J. Cartaud, E. L. Beneditti, J. B. Cohen, J. C. Meunier, J.-P. Changeux, *FEBS Lett.* **33**, 109 (1973); M. A. Raftery *et al.*, in *Protein Ligand Interactions*, H. Sund and C. Glauer, Eds. (de Gruyter, Berlin, 1978), p. 328; T. Allen, R. Baerwald, L. T. Potter, *Tissue Cell* **9**, 595 (1977).
27. M. Klymkowsky and R. M. Stroud, *ibid.* **128**, 319 (1979).
28. B. Conti-Tronconi, M. Morgutti, C. Gotti, F. Clementi, *Advances in Cytopharmacology*, B. Ceccarelli and F. Clementi, Eds. (Raven, New York 1979), vol. 3, p. 255.
29. W. M. Fitch and E. Margoliash, *Science* **155**, 279 (1967).
30. R. Poljak *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3305 (1973).
31. J. Boulter and J. Patrick, *Biochemistry* **16**, 4900 (1977); J. Lindstrom *et al.*, in (*12*); N. Nathanson and Z. Hall, *Biochemistry* **18**, 3392 (1979); A. Lyddiatt, K. Sumikawa, J. M. Wolosin, J. O. Dolly, E. A. Barnard, *FEBS Lett.* **108**, 20 (1979).
32. V. Damle, M. McLaughlin, A. Karlin, *Biochem. Biophys. Res. Commun.* **84**, 845 (1978); H.-P. Moore and M. A. Raftery, *Biochemistry* **18**, 1862 (1979).
33. A. T. Eldefrawi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2172 (1977); J. Elliot and M. A. Raftery, *Biochem. Biophys. Res. Commun.* **77**, 1347 (1977).
34. M. Weber and J.-P. Changeux, *Mol. Pharmacol.* **10**, 15 (1974); J. P. Cohen, M. Weber, J.-P. Changeux, *ibid.*, p. 904; M. Schimerlik and M. A. Raftery, *Biochem. Biophys. Res. Commun.* **73**, 607 (1976); S. G. Blanchard, J. Elliott, M. A. Raftery, *Biochemistry* **18**, 5580 (1979).
35. M. Kasai and J. -P. Changeux, *J. Membr. Biol.* **6**, 1 (1971); D. G. Clark, D. O. MacMurchie, E. Elliot, R. G. Wolcott, A. Landel, M. A. Raftery, *Biochemistry* **11**, 1663 (1972); G. P. Hess and J. P. Andrews, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 482 (1977); D. Miller, H. P. H. Moore, P. R. Hartig, M. A. Raftery, *Biochem. Biophys. Res. Commun.* **85**, 632 (1978).
36. P. R. Hartig and M. A. Raftery, *Biochem. Biophys. Res. Commun.* **78**, 16 (1977).
37. R. Tarrab-Hazdai, S. Geiger, S. Fuchs, A. Amsterdam, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2497 (1978); C. Strader, J.-P. Revel, M. A. Raftery, *J. Cell Biol.* **83**, 499 (1979).
38. Supported by PHS grants NS 10294 and GM-06965 and by a grant from the Muscular Dystrophy Association of America.

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Probing the Component Structure of a Maize Gene with Transposable Elements

Abstract. *Three instances of* R *gene instability were found in maize stocks carrying the controlling elements Dissociation (*Ds*) and Modulator (*Mp*). In each,* Ds *or a* Ds*-like element had transposed to* R*, inhibiting kernel pigmentation irregularly. When* Mp *was removed from the genome,* R *expression stabilized at low to intermediate levels. Strong pigmenting action was restored through recombination in heterozygotes of the three new forms with an* R *allele that specifies only plant pigmentation. The sites of* Ds *insertion mapped distal to the region that specifies seed versus plant expression. The evidence suggests that an* R *functional unit consists of one component that both governs tissue-specific expression and another that is common to alleles of different tissue-specific activities.*

Controlling elements are mobile units in the maize (*Zea mays*) genome that alter the expression of standard genes. The behavior of a number of different controlling elements, some acting singly and others as interacting pairs, has been worked out in detail (*1*). In the present study, controlling elements were used to probe the organization of a maize gene. The approach is analogous to the use made of transposable insertion sequences in *Escherichia coli* for determining the arrangement of ribosomal protein genes in operons (*2*).

The target locus *R* is one of a series of genes required for anthocyanin (flavonoid pigment) formation. The *R* alleles collected from diverse sources differ in distribution, intensity, and time of pigment formation (*3*). A given allele may comprise one or more units of independent function. Stadler (*4*) designated these units as genic elements since the individual elements of compound *R* alleles behave as separate genes. It has been shown that a number of alleles composed of multiple elements carry duplicated chromosome segments (*5*). An allele consisting of a single genic element was chosen for the present study in order to avoid the complexities attending chromosome rearrangement and multiple *R* elements. This allele, *R-sc:124*, specifies intense pigmentation both in the outer layer of the seed's endosperm (the aleurone) and the scutellum of the embryo (*6*). (Vegetative parts of the plant are not colored.) The element is designated (Sc) for self-colored, denoting the solid appearance of kernels. It contrasts with elements that effect pigmentation either in plant tissues exclusively or in some combination of kernel and plant tissues (*7*).

Are there, in addition to components that distinguish *R* elements, ones that are not tissue-specific in their effect? The latter might code, for example, for a product affecting a given step in pigment synthesis in any plant or seed part. Such a component would have gone undetected if it were invariant in the alleles studied so far.

Mutations in *R* were induced in a search for components whose effects are not tissue-specific. The general plan was to establish variants of a given *R* element that are of null or low-level activity and then to map the site of mutation relative to the site of the tissue-specific com-

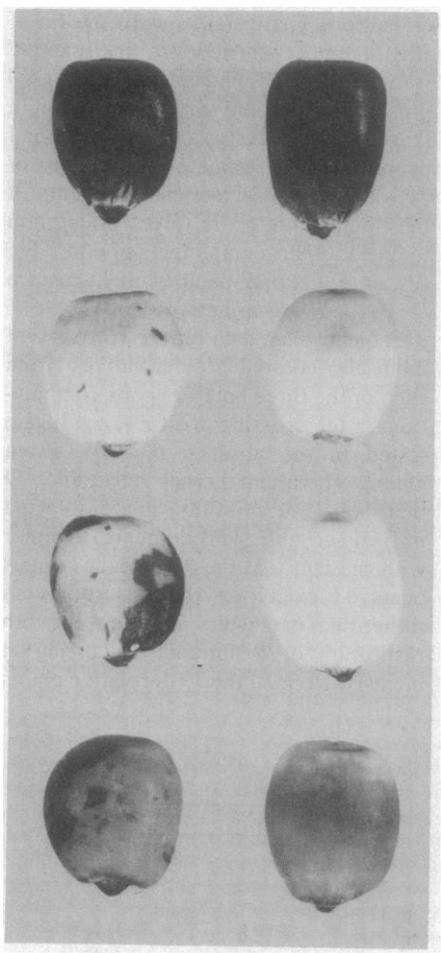

Fig. 1. Variations in aleurone pigmentation of maize endosperm due to controlling element action at the *R* locus. The *R* alleles are parental *R-sc* (top row) through *r*-mutable phenotypes m1, m3, and m9 (bottom row). In the left column, *Mp* is present in the genome; in the right column it is absent. (Pericarps have been removed.)

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Table 1. Strongly pigmented *R* derivatives isolated after testcrosses of *G r-m m-st/g r-r M-st* females with *g r-g m-st* males in the absence of *Mp* [*r-r* represents (P)(s) cases n35 and n101].

Source of *r-m*	Kernel population	Strong seed-color derivatives				
		Frequency ($\times 10^{-4}$)	Linked marker composition			
			G m-st	*g M-st*	*G M-st*	*g m-st*
ml	87,100	5.5	3	0	45	0
m3	41,350	6.5	0	0	27	0
m9	6,370	6.3*	0	0	4	0

*Minimal estimate. Some variants could have been undetected since the expression of parental m9 ranges through dark pale.

ponent in that element. The first positive results were obtained by utilizing variants induced by controlling elements. The rationale for using controlling elements was based on evidence that they simulate the effect of mutagens by insertion (*8*).

The transposable element Dissociation (*Ds*) was mobilized from an initial position proximal to the *waxy* gene in the short arm of chromosome 9 by hybridizing with a strain carrying Modulator (*Mp*), a component of the variegated pericarp gene *P-vv* (*9*). Both parental stocks were homozygous for the *R-sc:124* allele of *R* in the long arm of chromosome 10. Two of the five heritable variants of *R-sc* that were established after large-scale crossing of the hybrid with a stock lacking pigment in both seed and plant (*r-g*) behaved as would be expected if the alteration were caused by *Ds* insertion at *R*. In the absence of *Mp*, a uniformly pale (ml) or colorless (m3) phenotype was observed; in its presence, intensely pigmented areas on the pale or colorless background and occasional whole-kernel revertants occurred (see Fig. 1 and cover). Like receptor action in other mutable systems, *Ds* inhibition of *R-sc* in one chromosome was found not to interfere with expression of the allele in the homologous chromosome. Thus the action of *Ds* on *R* is cis-specific, whereas *Mp* incites ml and m3 instability in trans combination. A third *R* instability (m9) behaved similarly although it was isolated from an *R-sc P-vv* stock not known to carry *Ds*.

Stability of *Ds* at *R-sc* in ml and m3 and of the *Ds*-like element in m9 was tested by screening for intensely pigmented kernels after pollinating homozygous strains of the three variants with *r-g r-g*, both parents lacking *Mp*. No revertant to strong *R-sc* expression was obtained from ml, m3, or m9 in testcross populations numbering 41,010, 18,170, and 12,950 kernels, respectively. This result indicates that *Ds* is fixed at *R* in the absence of *Mp*, just as *Ds* has proved to be germinally stable at other loci after removal of the regulator from the genome (*8*, *10*).

Each of the three variants was studied in heterozygotes to test whether *R-sc* function could be restored by recombinational substitution of components from an *R* allele of contrasting tissue-specific effects. The contrasting allele class chosen descends from the *R* duplication chromosome *R-r:standard*. The proximal member of the duplication carried the genic element (P), so designated because of its positive effect on plant pigmentation. The distal (toward the tip of 10L) member carried (s), an inactive form of the seed-pigmenting element (S) present in *R-r:standard*. No reversion in these (P)(s) stocks to colored seed had occurred in a gamete population of 71,100 (*11*). Since member segments of the duplication are in direct orientation to one another, components of either the (P)- or (s)-bearing member might replace *Ds* in the *R-sc* variants.

Table 1 gives data on the recovery of intensely colored *R* derivatives from each of the three heterozygous combinations after crossing with *r-g r-g* (again tested in the absence of *Mp*). From among 91 variant kernel selections, 79 that were successfully progeny-tested proved heritable. This outcome, together with the fact that the 91 deviant kernels occurred singly on the testcross ears rather than in clusters, is consistent with restitution of *R* function occurring at meiosis. None of the 79 derivative plants developed another pigment, a characteristic feature of (P) action. When crossed to a colorless seed tester, each pigmented the seed uniformly as the original *R-sc:124* stocks and unlike the irregular (mottled) distribution characteristic of (S) action in (P)(S), the parent allele of (P)(s). A detailed study to compare pigmentation level in the 79 derived *R-sc*'s with the original *R-sc* stocks has not been made.

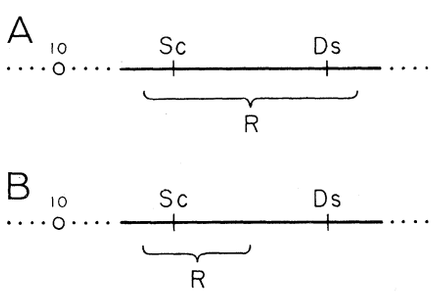

Fig. 2. Alternative interpretations of the position of *Ds* in *r*-mutable phenotypes ml, m3, and m9. (A) *Ds* acts by inserting within the region involved in the normal expression of *R*. (B) *Ds* lies outside the *R* functional unit.

To associate restoration of *R-sc* function with recombination in the *R* region, the flanking markers golden plant (*g*, 20 map units proximal) and Modifier of R-stippled (*M-st*, six units distal) had been incorporated into the (P)(s) chromosome. The relation between *R-sc* restoration and recombination of the flanking markers proved unexpectedly regular. Each of the 79 *R-sc* derivatives was borne on a chromosome carrying the green plant (*G*) allele of the golden locus from the *R-sc* variant parent; all but three carried *M-st* from the (P)(s) parent. Thus 76 of the 79 derivatives constituted one recombinant class and three one parental class, whereas the two remaining marker combinations were not represented. The relationship of markers in the recombinant class associates restoration of *R-sc* function with crossing over distal to (Sc). In such crossovers, the *Ds*-carrying segment of *R-sc* is evidently exchanged for a segment from the (P)(s) chromosome that is capable of supporting normal *R-sc* function.

A search for the reciprocal product of crossovers that restore *R-sc* action was made in the case of (P)(s) heterozygotes with m9. If the receptor element affecting (Sc) can be transferred to (P), thereby inhibiting its expression, the colorless kernel class should yield occasional green or weakly pigmented seedlings that are distinguishable from the parental red seedling class. A population of 2908 seedlings yielded three heritable variants. One lacked pigment in both plant and seed. It was borne on a chromosome marked as parental (P)(s), and it proved stable in the presence of *Mp*. The remaining two variants are characterized phenotypically by colorless seeds and weak seedling and anther pigmentation. Both were borne on recombinant chromosomes. Significantly, the marker combination (*g m-st*) is reciprocal to the four instances of *R-sc* restitution involving m9. When combined with *Mp*, the two displayed a mosaic of light and dark anther coloration. These characteristics indicate that the m9 receptor had been transferred from the *R-sc* chromosome to (P) by crossing over.

What is implied about *R* gene organi-

zation by the finding that *Ds* in m1, m3, and m9 had inserted closely distal (*12*) to the site that gives (Sc) its distinctive tissue-specific effect? One possibility is that the controlling element receptor may act by insertion within the affected gene (*13*). If so, *Ds* acts by inserting within an essential *R* component or between components whose function depends on a contiguous cis relationship (Fig. 2A). Crossover restitution of (Sc) function would involve replacing *Ds* by a segment from an *R* element in the homologous chromosome. This interpretation implies the presence of a segment of equivalent effect in different *R* genic elements. Components common to different *R* elements have not been disclosed by other means.

Alternatively, *Ds* may exert a regional effect in chromosome 10, inhibiting (Sc) action when inserted beyond the bounds of the *R* element (Fig. 2B). In this case, the crossover event that exchanges *Ds* between homologs need not involve components common to different *R* elements. This alternative would be ruled out for instances of *Ds* insertion that map between the sites of recessive *r* mutations induced by conventional means. Such data are not available for *R*. Comparable data have been obtained, however, through fine structure analysis of the *waxy* gene (*8*); three controlling element mutants mapped within the region delimited by recessive mutation that had occurred spontaneously or had been induced by gamma rays. A highly localized effect of controlling elements, such as is indicated by the data for the *waxy* gene, is clearly necessary if they are to resolve components within an *R* element. In inter se combinations, the three *waxy* variants recombined with one another. Again, the property of insertion at various sites on a local scale is essential if the present approach is to yield a representative picture of the *R* gene.

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References and Notes

1. Reviewed in J. R. S. Fincham and G. R. K. Sastry [*Annu. Rev. Genet.* **8**, 15 (1974)] and P. A. Peterson [in *Maize Breeding and Genetics*, D. B. Walden, Ed. (Wiley, New York, 1978), p. 601].
2. S. R. Jaskunas and M. Nomura, in *DNA Insertion Elements, Plasmids and Episomes*, A. I. Bukhari *et al.*, Eds. (Cold Spring Harbor Laboratory, Cold Water Harbor, N.Y., 1977), p. 487.
3. L. J. Stadler, *Cold Spring Harbor Symp. Quant. Biol.* **16**, 49 (1951); E. D. Styles, O. Ceska, K. T. Seah, *Can. J. Genet. Cytol.* **15**, 59 (1973).
4. L. J. Stadler, *Science* **120**, 811 (1954).
5. ——— and M. G. Neuffer, *ibid.* **117**, 471 (1953); L. J. Stadler and M. H. Emmerling, *Genetics* **41**, 124 (1956); J. L. Kermicle, *ibid.* **64**, 247 (1970); H. K. Dooner and J. L. Kermicle, *ibid.* **67**, 427 (1971); *ibid.* **82**, 309 (1976).
6. K. S. McWhirter and R. A. Brink, *Genetics* **47**, 1053 (1962).
7. The *R* alleles used in the present analysis and their probable genic element compositions are as follows: *R-sc:124* = (Sc), *R-r:standard* = (P)(S), *r-r:n35* & *r-r:n101* = (P)(s) and *r-g* = (p) or (s).
8. O. E. Nelson, *Genetics* **60**, 509 (1968).
9. B. McClintock, *Cold Spring Harbor Symp. Quant. Biol.* **16**, 13 (1951); R. A. Brink and R. A. Wilson, *Genetics* **37**, 519 (1952); P. C. Barclay and R. A. Brink, *Proc. Natl. Acad. Sci. U.S.A.* **40**, 1118 (1954).
10. M. G. Neuffer, *Genetics* **52**, 521 (1965).
11. H. K. Dooner and J. L. Kermicle, *ibid.* **78**, 691 (1974).
12. The percentage of recombination between (Sc) and *Ds* in m1, for example, may be calculated from the data in Table 1 as (45/87,100) × 100 × 2 = 0.10. The factor 2 enters in because only one of the two products of recombination is considered.
13. P. Nevers and H. Saedler, *Nature (London)* **268**, 109 (1977).
14. Paper 2396 from the Laboratory of Genetics. Conducted at the Wisconsin Agricultural Experiment Station with funds from DOE contract EY-76-S-02-1300.

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An About 50,000-Dalton Protein in Adrenal Medulla: A Common Precursor of [Met]- and [Leu]Enkephalin

Abstract. *A protein that may be an enkephalin precursor has been identified in extracts of bovine adrenal medulla. This protein (about 50,000 daltons) appears to contain seven copies of [Met]enkephalin and one copy of [Leu]enkephalin. Digestion with trypsin and carboxypeptidase B yields [Met]enkephalin and [Leu]enkephalin in a ratio of almost 7 to 1. The enkephalins were identified by chromatography and by their binding to opiate receptors. Some characteristics of several other adrenal peptides that may serve as intermediates in the biosynthesis of the enkephalins are presented.*

Demonstration of the [Met]enkephalin sequence (Try-Gly-Gly-Phe-Met) (*1*) at the amino terminus of β-endorphin (about 3000 daltons) (*2*) was originally interpreted as being indicative of a precursor-product relationship. However, attempts to demonstrate the presence of β-endorphin in tissues rich in enkephalins other than the pituitary gland, such as striatum (*3, 4*) and adrenal medulla (*5*), have been unsuccessful. In contrast, from extracts of adrenal medulla we were able to isolate a number of small (*6*) and large (*7*) enkephalin-containing peptides ranging from 500 to 22,000 daltons (*8*). In those studies, the enkephalin-like activities in the isolated peptides and proteins were revealed after tryptic digestion by their ability to compete with tritiated [Leu]enkephalin for binding to neuroblastoma-glioma cells; the released peptides were identified by high-performance liquid chromatography (*9*). The presence of free enkephalin in the tryptic digests indicates that the pentapeptide sequence occurred at the carboxyl terminus; hexapeptides with Arg or Lys in position 6 were found when the enkephalin sequence occurred internally. Since enkephalins extended at the carboxyl terminus with Arg or Lys possess very weak receptor binding activity (*10*), their detection and quantification in tryptic digests of peptides

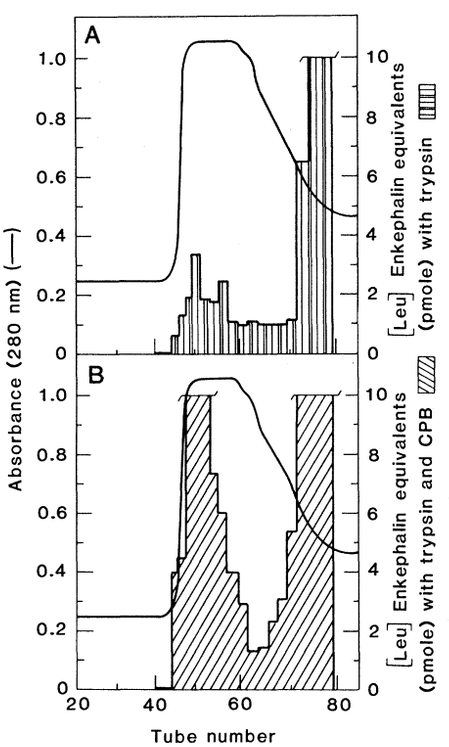

Fig. 1. Chromatography of extracts of adrenal medulla on Sephadex G-75. Bovine adrenal medulla (26 g) were homogenized in 100 ml of 1*M* acetic acid, 20 m*M* HCl, 0.1 percent β-mercaptoethanol containing phenyl methane sulfonyl fluoride (PMSF) (1 μg/ml) and pepstatin (1 μg/ml). The extract was centrifuged at 140,000*g* for 60 minutes. The supernatant was chromatographed on Sephadex G-75 (5 by 100 cm) with the above buffer and without PMSF and pepstatin. Portions (200 μl) from each fraction were lyophilized, redissolved in 400 μl of 50 m*M* tris-HCl (*p*H 8.5), and digested with 1 μg of TPCK-treated trypsin for 16 hours at 37°C. Tryptic digests (200 μl) were treated with 0.1 μg of carboxypeptidase B (*CPB*) for 2 hours at 37°C and then for 20 minutes at 90°C to inactivate the enzyme. Each portion was assayed with the neuroblastoma-glioma cell binding assay (*9*), with trypsin (A), and with trypsin and carboxypeptidase B (B). The activity in tubes 48 to 54 represents the 50,000-dalton protein and the activity after tube 66 represents the beginning of the 22,000-dalton protein.

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