TWO-GENE CONTROL OF THE EXPRESSION OF A MURINE Ia ANTIGEN*

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The *I* region of the *H-2* major histocompatibility complex of the mouse contains a series of loci involved in the induction and regulation of immune responses (1, 2). This segment of the *H-2* complex was initially demarcated by several loci, the immune response (*Ir*) genes,[1] which control the capacity to mount specific immune responses to certain antigens (3). The ability to respond to an antigen under *Ir* gene control is a dominant Mendelian trait; some alleles but not others confer responsiveness to that antigen (4). Responsiveness to some antigens has been shown more recently to be under the control of two *Ir* loci; appropriate nonresponder haplotypes combined either in heterozygotes or in intra-*I* region recombinant strains are able to complement each other to allow a response (5, 6). Similar two-gene control of the generation of antigen-specific suppression by immune suppression (*Is*) loci has also been well documented (6).

Subsequent to the discovery of *Ir* genes, sets of alloantigens also controlled by loci mapping in the *I* region were described (1). These *I* region associated (Ia) antigens are prominent on lymphocytes and have also been detected on macrophages and epidermal cells (7, 8). Lymphocyte Ia antigens are highly polymorphic glycoproteins with mol wt ranging from 25,000 to 35,000 daltons (9). It has been suggested that Ia antigens are the products of the *Ir* genes; as cell surface molecules they might regulate responsiveness either by participating in antigen presentation and recognition (10, 11) or as cell interaction molecules mediating collaborations between B cells, T cells, and macrophages (12, 13). The molecular mechanisms of action of Ia antigens and their relevance to *Ir* gene function remain to be elucidated.

We have been analyzing the genetics, structure, and expression of Ia antigens with the hope that such studies will provide insights into the functions of these cell surface proteins. Ia antigens immunoprecipitated from detergent extracts of radiolabeled lymphocytes have been resolved by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) (14) into multiple species representing the products of several loci

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[1] *Abbreviations used in this paper:* Ia, *I*-region associated; IEF, isoelectricfocussing; *Ir* genes, immune response genes; *Is* genes, immune suppression genes; NEPHGE, nonequilibrium pH gradient electrophoresis; NP-40, Non-Idet P-40; PAGE, polyacrylamide gel electrophoresis; SaC, *Staphylococcus aureus*, Cowan I strain, absorbent; SDS, sodium dodecyl sulfate; 2-D, two-dimensionl.

within the *I* region (15, 16). In this study we demonstrate that the cell surface expression of some Ia antigens is under the control of two genes, both of which map between the *K* and *D* regions. If, in fact, Ia antigens do mediate *Ir* gene function, the phenomena described below suggest possible mechanisms by which two *Ir* genes might complement each other through their coordinate regulation of the expression of a molecular complex on the cell surface.

Materials and Methods

Mice. Heterozygous (B10 × B10.A)F_1, (B10.A(4R) × B10.A)F_1, and (B10.A(3R) × B10.A(4R))F_1 mice were generously provided by Dr. Jeffrey Frelinger, University of Southern California. Other mice were raised in our own animal facilities.

Isolation of Radiolabeled Ia Antigens. The procedures for radiolabeling mouse spleen lymphocytes and for extracting and immunoprecipitating Ia antigens have been previously described (15). Briefly, splenic lymphocytes were incubated for 4–5 h at 2.5 × 10^7 cells/ml in methionine-free Eagle's medium containing 5% fetal calf serum and 250 μCi/ml [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill. 600 Ci/mM). Membrane proteins were extracted from washed cells with 0.5% Non-Idet P-40 (NP-40). Ia antigens were immunoprecipitated from the extracts with specific alloantisera, prepared as described elsewhere (17), followed by the addition of heat killed and formalin-fixed *Staphylococcus aureus*, Cowan Strain I (SaC), which bind the antigen-antibody complexes (18). The bound proteins were eluted from the SaC with isoelectric-focussing (IEF) sample buffer (15).

2-D PAGE. Lymphocyte proteins were separated in two dimensions according to the method introduced by O'Farrell (14). Two different electrophoretic techniques which separate proteins according to their charge were used for the first dimension electrophoresis in tube gels. The first, IEF, was performed as described by O'Farrell (14). The second procedure, based on an approach developed by O'Farrell et al. (19) is nonequilibrium pH gradient electrophoresis (NEPHGE) which allows resolution of basic cell proteins which do not focus in the IEF gels. The NEPHGE tube gels are prepared as described for IEF gels (14) except that the ampholytes are made up entirely of pH range 3.5–10 ampholines (LKB Instruments, Inc., Rockville, Md.) instead of a mixture of pH 3.5–10 and pH 5–7 ampholines. Samples are electrophoresed on NEPHGE gels towards the cathode for 6 h at 500 V, conditions which allow maximum separation and resolution of both acidic and basic Ia antigens (P. Jones, unpublished observations).

The first dimension IEF or NEPHGE tube gels are equilibrated in sodium dodecyl sulfate (SDS) sample buffer, and then for the second dimension they are imbedded on top of 10% acrylamide SDS slab gels as described by O'Farrell (14). The slab gels are fixed and stained, dried, and exposed to Kodak NS2T No Screen X-ray film for autoradiography (14, 15).

Results

Immunoprecipitable Ia Antigens. In earlier studies we established that antisera directed against products of loci mapping in the *I-A* or *I-E* subregions readily precipitate Ia antigens from NP-40 extracts of radiolabeled spleen lymphocytes (15, 16).[2] When resolved on 2-D gels, these immunoprecipitates generate patterns that are both haplotype-specific and characteristic of the *I-A* or *I-E* subregion.

One of the antisera used previously to precipitate $I\text{-}E^k$ products (16), (B10 × HTI)F_1 anti-B10.A(5R), was used in the experiments reported below. As shown in Table I, this antiserum potentially can react with products of loci mapping in the $I\text{-}J^k$, $I\text{-}E^k$, $I\text{-}C^d$, S^d, and G^d regions. In the precipitation assay used here, it is probable that the only reactivity detected is against $I\text{-}E^k$ subregion product(s). The *S* and *G* regions do not code for proteins detected on lymphocytes (1, 20). I-J antigens are expressed on only a small population of T lymphocytes (21), and thus far we have

[2] P. Jones, D. B. Murphy, and H. O. McDevitt. Manuscript in preparation.

TABLE I

Reactivity of the (B10 × HTI)F_1 Anti-B10.A(5R) Antiserum

			I							
		K	*A*	*B*	*J*	*E*	*C*	*S*	*G*	*D*
Antiserum producer:	(B10	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
	× HTI)F_1	*b*	*b*	*b*	*b*	*b*	*b*	*b*	?	*d*
Cell donor:	B10.A(5R)	*b*	*b*	*b*	*k*	*k*	*d*	*d*	*d* *	*d*

* The box indicates the potential reactivities of this antiserum.

been unable to detect any proteins precipitated by anti-I-J antisera (P. Jones and D. B. Murphy, unpublished observations). Finally, we have not been able to detect by immunoprecipitation any Ia determinants controlled by loci mapping in *I-C*; a number of antisera with potential reactivity against both *I-E* and *I-C* do not precipitate more molecules than do antisera that can react with *I-E* but not *I-C* (16). Therefore, it seems likely that the (B10 × HTI)F_1 anti-B10.A(5R) serum detects I-E molecules only.

Some Molecules Precipitated by Anti-I-E^k Antibodies from Recombinant Strain B10.A(3R) Extracts are Different from Those Obtained from B10.A Extracts. A typical 2-D gel pattern generated by the molecules precipitated from an NP-40 extract of [^{35}S]methionine-labeled B10.A (*H-2^a*) spleen cells by the (B10 × HTI)F_1 anti-B10.A(5R) serum (anti-I-E^k) is shown in Fig. 1 a. From a comparison of Fig. 1 a with Fig. 1 c, the background spots brought down nonspecifically can be identified. (B10 × HTI)F_1 anti-B10.A(5R) does not react with molecules from B10 (*H-2^b*) since the *b* haplotype is present in the recipient used for antiserum production (Fig. 1 c), and normal mouse serum control precipitates give identical patterns (not shown). Among the proteins brought down nonspecifically in all precipitates are actin (indicated by the letter a in Fig 1 a) and γ-chains from IgG synthesized by the spleen cells (visible as the dark streak in the upper left hand corner of each panel). In Fig. 1 a, the heterogeneous group of spots in the lower right are the polymorphic Ia antigens specifically precipitated by the antiserum. The major spot on the left, indicated by the letter i, is a very basic nonpolymorphic polypeptide chain, mol wt 31,000, which is present in all Ia immunoprecipitates. The properties of this invariant chain will be discussed elsewhere.[2]

The pattern shown in Fig. 1 a for B10.A is identical to the pattern obtained from seven other mouse strains which express I-E^k determinants, summarized in Table II, lines 3–7. However, a different pattern is generated by the immunoprecipitates obtained with the same anti-I-E^k anti-serum from B10.A(3R) and B10.A(5R) (referred to as 3R and 5R, respectively) which are recombinants derived from (B10 × A)F_1 heterozygotes (*H-2^b/H-2^a*) (Table II). The pattern formed by the precipitate from 3R (Fig. 1 b) is similar to that of B10.A, but spots 1′, 2′, and 3′ clearly are shifted to left compared to the analogous spots 1, 2, and 3 in the B10.A gel. Aside from the spot indicated with a question mark, which seems to be darker for 3R, the remaining spots seem to correspond in position to those of B10.A. The Ia antigens shared between 3R and B10.A are most likely controlled by the *I-E^k* subregion, as discussed in the preceding section. The 5R recombinant gives a gel pattern (not shown) identical to that of 3R.

Thus, two *H-2^b/H-2^a* recombinant strains give an I-E^k gel pattern which is, in part,

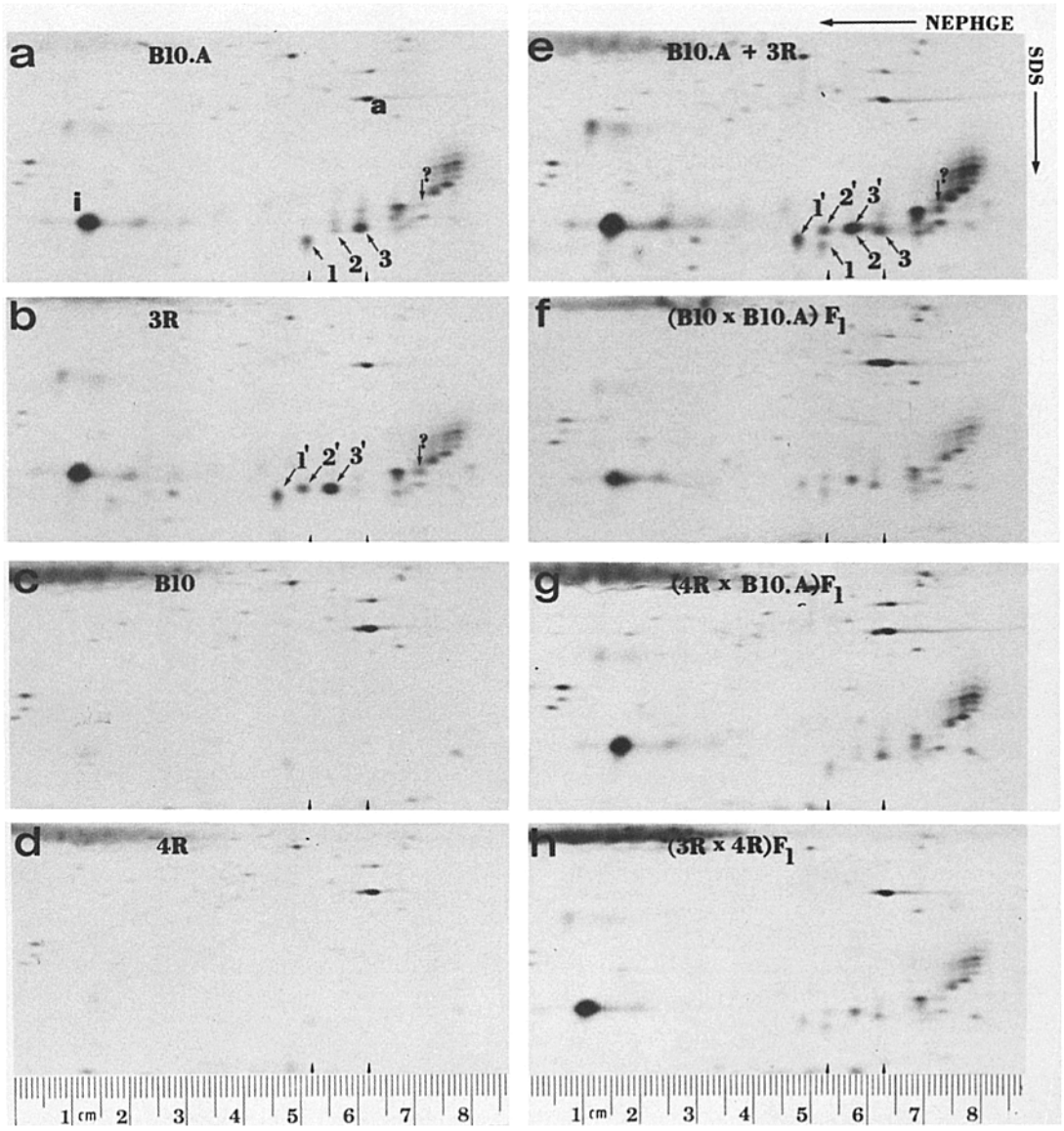

FIG. 1. NEPHGE 2-D gels of anti-I-E^k immunoprecipitates. Immunoprecipitates were prepared by reacting 100 μl 0.5% NP-40 extract (obtained from 10^7 cells) with 30 μl (B10 × HTI)F_1 anti-5R antiserum, followed by 200 μl of 10% suspension of SaC. Radiolabeled proteins were eluted from the SaC with 50 μl of IEF sample buffer (14) and 20 μl were loaded per gel. For e, 20 μl each of the B10.A and 3R samples shown in a and b were mixed and coelectrophoresed. The amount of ^{35}S radioactivity in the samples ranged from 20,000 to 45,000 cpm. To equalize the intensity of the spots as much as possible from gel to gel, the length of autoradiographic exposure was varied from 8 to 23 days. The gels shown here were taken from several experiments; each gel has been repeated in at least three independent experiments, giving similar results.

The first dimension NEPHGE gels and second dimension SDS gels were run in the indicated directions. The basic (pH 9.0) end is on the left and the acidic (pH 4.5) end is on the right (19). Each panel represents the portion of the autoradiogram containing proteins ranging in size from 25,000 to 55,000 daltons. Actin, indicated by the letter a is 44,000 daltons. The invariant Ia polypeptide chain, indicated by the letter i, is 31,000 daltons. The Ia spots labeled with numbers are discussed in the text. The autoradiograms were positioned so that the actin spots are aligned, as indicated by the mark at the bottom of each panel corresponding to position 6.2 for a–d, 6.3 for e–h. The other mark, at position 5.2 for a–d, 5.3 for e–h, indicates the average position of spot 1.

TABLE II

Haplotypes and 2-D Gel Patterns of I-E^k Immunoprecipitates of Strains Used In This Study

Strain	Haplotype	Genotype* I									2-D Gel pattern of I-E^k immunoprecipitates‡	
		K	*A*	*B*	*J*	*E*	*C*	*S*	*G*	*D*	B10.A-like	B10.A(3R)-like
1. C57BL/10, C57BL/6, C3H.SW	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	−	−
2. HTI	*il*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	?	*b*		ND§
3. B10.BR, C3H, AKR	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	+	−
4. A.TL	*tl*	*s*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*d*	+	−
5. B10.A, A/J	*a*	*k*	*k*	*k*	*k*	*k*	*d*	*d*	*d*	*d*	+	−
6. B10.A(1R), B10.A(2R)	*h1,h2*	*k*	*k*	*k*	*k*	*k*	*d*	*d*	?	*b*	+	−
7. B10.AQR	*y1*	*q*	*k*	*k*	*k*	*k*	*d*	*d*	*d*	*d*	+	−
8. B10.A(4R)	*h4*	*k*	*k*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	−	−
9. B10.A(3R)	*i3*	*b*	*b*	*b*	*b*	*k*	*d*	*d*	*d*	*d*	−	+
10. B10.A(5R)	*i5*	*b*	*b*	*b*	*k*	*k*	*d*	*d*	*d*	*d*	−	+
11. (B10 × B10.BR)F$_1$, (B10 × AKR)F$_1$	*b/k*	*b/k*	*b/k*	*b/k*	*b/k*	*b/k*	*b/k*	*b/k*	*b/k*	*b/k*	+	+
12. (B10 × B10.A)F$_1$	*b/a*	*b/k*	*b/k*	*b/k*	*b/k*	*b/k*	*b/d*	*b/d*	*b/d*	*b/d*	+	+
13. (B10.A(3R) × B10.A(4R))F$_1$	*i3/h4*	*b/k*	*b/k*	*b/b*	*b/b*	*k/b*	*d/b*	*d/b*	*d/b*	*d/b*	+	+
14. (B10.A(4R) × B10.A)F$_1$	*h4/a*	*k/k*	*k/k*	*b/k*	*b/k*	*b/k*	*b/d*	*b/d*	*b/d*	*b/d*	+	−

* Haplotype designations according to Murphy et al. (21), David (22), and Shreffler et al. (23).

‡ Immunoprecipitates were obtained from NP-40 extracts of spleen cells using (B10 × HTI)F$_1$ anti-B10.A(5R), which detects I-E antigens in this assay (see text). The B10.A-like and B10.A(3R)-like 2-D gel patterns are defined in Figs. 1 a and 1 b, respectively.

§ ND, not done.

different from that observed with parental strain B10.A. As will be discussed later, the spots within each group (i.e., 1, 2, 3 and 1′, 2′, 3′) appear to represent different modified forms of a single polypeptide chain. However, the shifts in position of these spots between B10.A and 3R or 5R cannot be accounted for simply on the basis of a mutation in the structural gene for this chain, unless one postulates that the same mutation occurred in an *I-E^k* subregion locus in both recombinant strains subsequent to their derivation, a most unlikely event. Rather, the new spot pattern would appear to be due to interactions between two loci situated on opposite sides of the cross-over positions in 3R and 5R. For example, recombinant strains 3R and 5R derived the K-end of their *H-2* complex from B10 (*H-2^b*) and their D-end from A (*H-2^a*) (Table II). Interplay between an *H-2^b*-derived locus and an *H-2^a*-derived locus in the *cis* chromosomal position could generate spots 1′, 2′, and 3′ in strains 3R and 5R. Data presented in the next three sections support this alternative and show further that (*a*) the interaction between these loci can occur in either the *cis* or *trans* chromosomal position, and (*b*) one locus maps in the *I-A* subregion, the second in *I-E, I-C, S,* or *G.*

The New Ia Molecules Found in 3R Extracts are also Present in (b × a)F$_1$ and (b × k)F$_1$ Extracts. Anti-I-E^k precipitates obtained from (B10 × B10.A)F$_1$ extracts exhibit a pattern which contains the same spots (1′, 2′, and 3′) found with 3R extracts, in addition to spots 1, 2, and 3 derived from the B10.A parent (Fig. 1 f). Indeed, the pattern obtained with F$_1$ extracts is identical (though reduced in intensity) to the configuration obtained by mixing B10.A and 3R immunoprecipitates together before electrophoresis (Fig. 1 e). As was observed with 3R and 5R, these precipitates again show an I-E^k gel pattern which is, in part, different from that observed with the parental B10.A strain, and strongly suggest that the new spots observed are not due to mutation. Identical spot patterns were also found in precipitates from the (*b* × *k*)F$_1$ heterozygotes (B10 × B10.BR)F$_1$ and (B10 × AKR)F$_1$ (Table II, line 11). These data reveal that interaction between an *H-2^b* derived locus and an *H-2^a* or *H-2^k* derived locus resulting in the new 3R-like gel pattern can occur when the two loci are

on different chromosomes (*trans* position) as well as on the same chromosome (*cis* position).

Further Evidence that Interaction between Two Loci in F_1 Heterozygotes can Generate New Ia Molecules not Precipitated from Either Parental Strain. The interaction between two loci in another *trans* combination is shown in Fig. 1 h. Crossing strain 4R (a third $H\text{-}2^a/H\text{-}2^b$ recombinant strain which carries the K-end from $H\text{-}2^a$ and the D-end from $H\text{-}2^b$) (Table II) with strain 3R results in the generation of new spots (1, 2, and 3) which are not precipitated from either parental strain. (Compare Fig. 1 h with 1 b and 1 d). In this particular case, interplay between two $H\text{-}2^a$-derived loci (one from 4R, the second from 3R) generates the new pattern. Note that these new spots are identical to those observed when the two loci are in the *cis* position as in B10.A and several other strains (Fig. 1 a, and Table II, lines 3–7).

Mapping the Two Loci which Control the New Ia Molecules. One locus controlling the new Ia molecules maps in the *I-A* subregion. Spots 1′, 2′, and 3′ are precipitated from (B10 × B10.A)F_1 extracts (Fig. 1 f) but not from (4R × B10.A)F_1 extracts (Fig. 1 g). Strain 4R differs from strain B10 only in the *K* and *I-A* regions (Table II), positioning this locus to the left of the *I-B* subregion. Furthermore, spots 1, 2, and 3 can be precipitated from both B10.A and B10.AQR, which differ only in that B10.AQR has $H\text{-}2K^q$ (Table II). Since spot 1 cannot be detected in extracts from the *q* haplotype strain B10.G, this spot is *k* haplotype in origin. Therefore, it can be concluded that one of the loci controlling spots 1, 2, and 3 maps to the right of the *K* region, in the *I-A* subregion.

The second locus controlling the new Ia molecules maps in *I-E, I-C, S,* or *G.* Spots 1′, 2′, and 3′ are precipitated from 3R extracts but not from B10 extracts, positioning the locus to the right of the *I-J* subregion (Table II). In addition, spots 1, 2, and 3 are precipitated from B10.A(1R) or B10.A(2R) extracts but not from B10 extracts, positioning the locus to the left of the *H-2D* region (Table II).

Thus, spots 1′, 2′, and 3′ are controlled by the interaction between a locus mapping in $I\text{-}A^b$ and a locus mapping in $I\text{-}E^k$, $I\text{-}C^d$, S^d, or G^d. In turn, spots 1, 2, and 3 are controlled by the interaction between a locus mapping in $I\text{-}A^k$ and a locus mapping in $I\text{-}E^k$, $I\text{-}C^d$, S^d, or G^d. The interactions which effect the electrophoretic mobilities of molecules precipitated by the anti-$I\text{-}E^k$ serum could be explained in one of two ways. One possibility is that a product of a locus mapping in *I-A* modifies in a haplotype-specific fashion a polypeptide chain coded for by a locus in *I-E.* For example, this could be achieved by addition of carbohydrate groups to an *I-E*-controlled polypeptide. Alternatively, the molecules whose positions are affected may actually be the product of a locus mapping in *I-A,* which is regulated by a locus mapping in *I-E, C, S,* or *G.* As has been shown above, all of these molecules are precipitated by an antiserum against $I\text{-}E^k$ which does not react with $I\text{-}A^b$ (Fig. 1 c) or $I\text{-}A^k$ (Fig. 1 d). Precipitation of the *I-A* product with the anti-*I-E* serum could occur if the *I-A* product was associated with the *I-E* product in a multi-subunit structure.

According to the second explanation, spots 1, 2, and 3 in B10.A represent the product of a locus mapping in $I\text{-}A^k$, while spots 1′, 2′, and 3′ in 3R represent the product of a locus mapping in $I\text{-}A^b$. The data which follow support this theory.

A Locus Mapping in I-A Codes for Molecules 1 and 1′. By examining 2-D gels of the NP-40 extracts themselves, without the use of antisera for immunoprecipitation, the locus coding for spots 1 and 1′ was mapped to *I-A.* Fig. 2 presents gels of NP-40

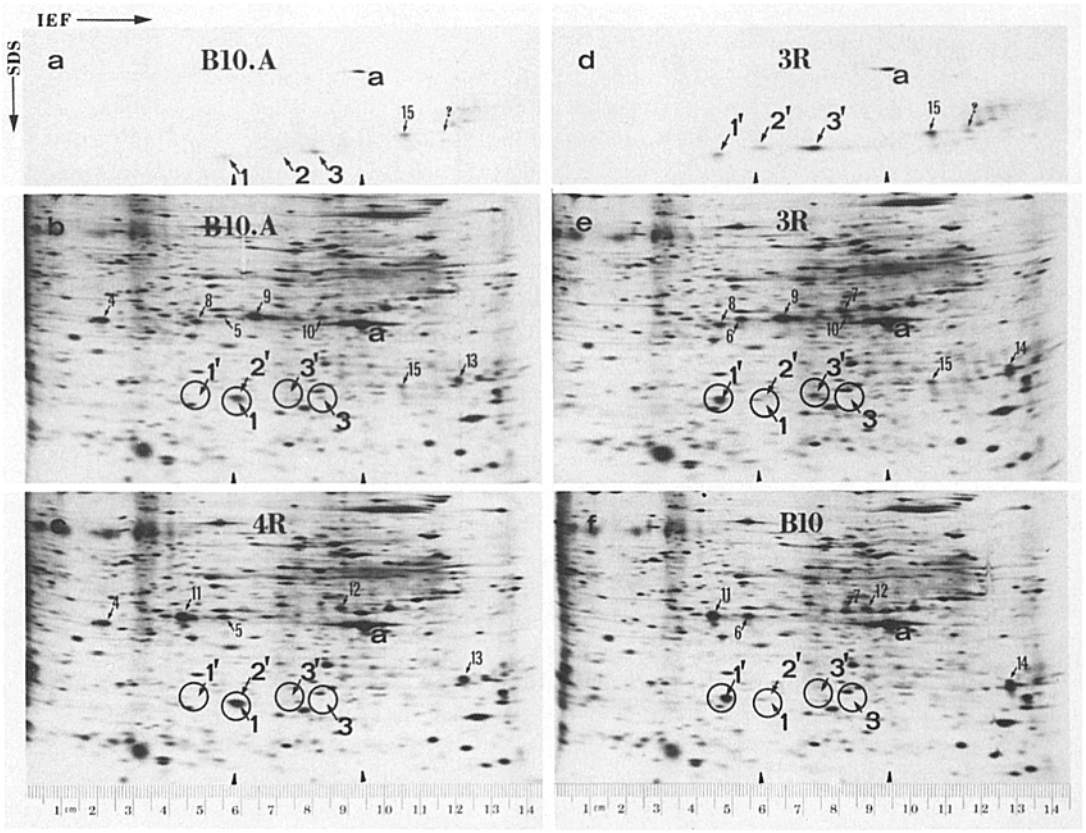

FIG. 2. IEF 2-D gels demonstrating that anti-I-E^k antibodies precipitate molecules coded for by both the *I-A* and *I-E* subregions. Immunoprecipitates obtained with (B10 × HTI)F_1 anti-5R antiserum as described in the legend to Fig. 1 are shown for a B10.A (26,000 cpm) and d 3R (30,000 cpm). The total cell proteins extracted by 0.5% NP-40 are shown for b B10.A, c 4R, e 3R, and f B10. Each extract sample contained 500,000 cpm of acid-insoluble radioactivity. The samples were prepared for isoelectrofocusing by adding crystalline urea to the extracts to a final concentration of 9 *M* followed by the addition of an equal volume of IEF sample buffer (15). The immunoprecipitate gels (a, d) were exposed to film for 14 days; the NP-40 extract gels (b, c, e, f) for 7 days. The gels shown here were all obtained in the same experiment; these results have been confirmed in several additional experiments.

The first dimension IEF gels and the second dimension SDS gels were run in the indicated directions; again the basic end of the gel (pH 7) is on the left and the acidic end (pH 4.5) is on the right (14, 15). Actin is indicated by the letter a. The spots labeled with large numbers represent the Ia molecules similarly labeled in Fig. 1; their positions in the extract gels are indicated with arrows and circles whether or not they actually are present. The spots labeled with small numbers represent other spots which differ among the four mouse strains (see text). The marks at the bottom of each panel indicate the positions of the actin spot (position 9.4) and of spot 1 (position 5.8).

extracts of B10.A, 3R, 4R, and B10 and for reference, the *I-E^k* precipitates obtained from B10.A and 3R with the (B10 × HTI)F_1 anti-5R antiserum used above. The 2-D gels shown in Fig. 2 were all obtained using IEF first dimension gels instead of NEPHGE gels. In general, unless the proteins of interest are very basic, IEF gels are used when the samples consist of total cell extracts because the resolution of spots from each other is better; this allows small mobility differences to be detected. However, as is evident in Figs. 2 a and 2 d, separation of immunoprecipitated proteins

in IEF gels tends to generate spots which streak and are less well resolved compared to NEPHGE gels (compare with Fig. 1).

The spots designated 1, 2, 3 and 1′, 2′, 3′ in the anti-I-E^k immunoprecipitates of B10.A and 3R (Figs. 2a and 2d) correspond to the similarly-labeled spots in Figs. 1a and 1b. By superimposing the autoradiograms of the B10.A and 3R immunoprecipitates on the autoradiograms of the total cell extracts from these strains, it was possible to identify unambiguously the corresponding spots in the extracts. For ease of reference, the positions of these spots in the extract gels are marked with arrows and circles, whether or not they actually are present in that particular extract. As anticipated, spots 1 and 3 are evident in the B10.A extract but not in the 3R extract (the faint spot in the position of spot 3 in Fig. 2e has not been observed in other 3R extracts). Spot 2 is very weak (Fig. 2a) and is not discernible in the extract, so it has not been numerically labeled. On the other hand, spots 1′, 2′, and 3′ are present in the 3R extract but not in the B10.A extract.

The key to understanding the subregion origin of spot 1 and 1′ lies in determining which spots are present in extracts from 4R and B10 (Figs. 2c and 2f). These strains differ only in that 4R has *H-2K*^{k} and *I-A*^{k}. Similar to strain B10.A, strain 4R has spot 1, whereas strain B10 has spot 1′, as does strain 3R. Therefore spots 1 and 1′ are controlled by a locus mapping in *H-2K* or *I-A*; spot 1 is the *k* haplotype product, while spot 1′ is the *b* haplotype form. As discussed earlier, an I-E^k gel pattern identical to that of B10.A is obtained from strain B10.AQR which differs from B10.A in the *K* region only (Table II line 7). Since extracts prepared from B10.G (which differs from B10.AQR in the *I, S, G,* and *D* regions) do not have spot 1, the locus controlling this molecule maps in the *I-A* subregion. Thus, spot 1 represents an I-A^k product, and spot 1′ represents an I-A^b product.

Molecules 1 and 1′ Appear to be Cytoplasmic Precursors of Molecules 2, 3, and 2′, 3′, Respectively. As shown in Fig. 2, 4R has spot 1, but it does not have spot 3, which is present in B10.A; similarly, B10 has spot 1′, but it is missing spots 2′ and 3′ which are unique to 3R. A number of observations have indicated that spots 1 and 1′ are cytoplasmic precursors of spots 2, 3 and 2′, 3′ respectively. Lactoperoxidase-catalyzed radioiodination of intact B10.A and 3R cells labels spots 2, 3, and 2′, 3′, but not spots 1 or 1′. In addition, a 15-min pulse with [^{35}S]methionine labels only the first spot in each of the groups, but during several hours of chase with cold methionine the label appears in spots 2, 3 and 2′, 3′ (P. Jones, unpublished observations). Similar results have been obtained with *d* haplotype Ia molecules (15). Thus, 4R and B10 seem to have the cytoplasmic precursors but not the cell surface forms of this *I-A* encoded protein.

A Locus Mapping in I-E, I-C, S, or *G Modifies the I-A Product, Which Then is Inserted into the Cell Membrane.* The appearance of spots 2′, 3′ in 3R must be controlled by a locus mapping to the right (*I-E, I-C, S, G,* or *D*) of the crossover in this strain, since these cell surface forms of spot 1′ are not present in strain B10. Similarly, the appearance of spots 2, 3 in B10.A must be controlled by a locus mapping to the left (*I-E, I-C, S,* or *G*) of the *D* region, since they are present in strains 1R and 2R (see Table II). More definitive mapping studies are in progress to localize the gene regulating the cell surface expression of the *I-A* polypeptide chain. The possible mechanism(s) by which the locus mapping in *I-E, I-C, S,* or *G* modifies the *I-A* product and the possible association of the modified *I-A* molecules with *I-E* controlled molecules will be considered in the discussion.

The Only Proteins Which Differ Among Extacts of B10.A, 4R, 3R, and B10 Are Products of the H-2 Complex. For the type of comparative analysis of total cell extracts used above to be valid and meaningful, the gels must show a high degree of reproducibility. The four autoradiograms shown in Fig. 2 meet this requirement. Aside from the spots already discussed, the only spots which differ discernibly from extract to extract have been shown by precipitation with specific alloantisera to be H-2 or Ia antigens (15, and P. Jones, unpublished results). The positions of these spots, marked with small numbers in Fig. 2, vary with the haplotype of each congenic strain at the appropriate locus. Thus spots 4 and 5, shared by B10.A and 4R are $H\text{-}2K^k$; spots 6 and 7, shared by 3R and B10 are $H\text{-}2K^b$; spots 8–10, shared by B10.A and 3R are $H\text{-}2D^d$; spots 11 and 12, shared by 4R and B10, are $H\text{-}2D^b$; spot 13 shared by B10.A and 4R is $I\text{-}A^k$, and spot 14, shared by 3R and B10, is $I\text{-}A^b$. Spot 15, shared by B10.A and 3R is probably $I\text{-}E^k$, as will be discussed below. Analogous results also have been obtained with the congenic strains C3H ($H\text{-}2^k$) and C3H.SW ($H\text{-}2^b$): C3H has spots 1, 2, and 3 and the appropriate $H\text{-}2K^k$ and $H\text{-}2D^k$ spots, while, similar to B10, C3H.SW has spots 1′ (but not 2′ or 3′) and the corresponding $H\text{-}2K^b$ and $H\text{-}2D^b$ spots (P. Jones, unpublished observations).

Discussion

The results presented above suggest that antisera directed against products of the *I-E* subregion of the *k* haplotype precipitate molecules coded for by loci in both the *I-A* and *I-E* subregions. Earlier studies, in fact, had indicated that the molecules precipitated from both *k* and *d* haplotypes with antibodies against *I-E* controlled antigens represented the products of two structural loci (15). For example, surface labeling of cells with ^{125}I, [^{35}S]methionine pulse chase experiments, and the treatment of radiolabeled cells with neuraminidase allowed the total array of spots precipitated by anti-I-E antibodies to be divided into two groups. In the first group, corresponding to spots 1–3, the first (most basic) spot appears to be the precursor of the other molecules (e.g., spots 2 and 3) which are cell surface forms. The second group consists of more acidic molecules; spot 15 or the spot directly under it (see Figs. 2a and 2d) appears to be the precursor for the other species in this series. In both groups, the molecules on the cell surface are larger and more acidic than their cytoplasmic precursors, as would be expected if the molecules are being modified by the addition of neutral sugars and sialic acid during their passage to the cell surface.

Results presented here support and extend these earlier findings. One locus, which maps in the *I-E* subregion, codes for spot 15 and its derivatives. The second locus, which maps in the *I-A* subregion, codes for spots 1 and 1′ and their modified forms. If, as seems likely, the $I\text{-}A^k$ spot 1 and the $I\text{-}A^b$ spot 1′ are the cytoplasmic precursors of the second and third spots, then the combination in either the *cis* or *trans* chromosomal position of the locus coding for these spots with a locus mapping between *I-J* and *H-2D* has two effects. First, it allows the modification of molecule 1 or 1′, generating molecules 2 and 3 or 2′ and 3′ which are expressed on the cell surface. Second, all of these $I\text{-}A^k$ or $I\text{-}A^b$ molecules, both cytoplasmic and cell surface forms, now are immunoprecipitable with antibodies directed against products of the $I\text{-}E^k$ subregion. These observations, coupled with the failure of the (B10 × HTI)F_1 anti-5R antiserum to specifically precipitate any Ia antigens from B10 or 4R, suggest that the $I\text{-}A^k$ and $I\text{-}A^b$ molecules precipitated by this antiserum from B10.A and 3R, respectively, are brought down because they are bound to molecules coded for by *I-E*.

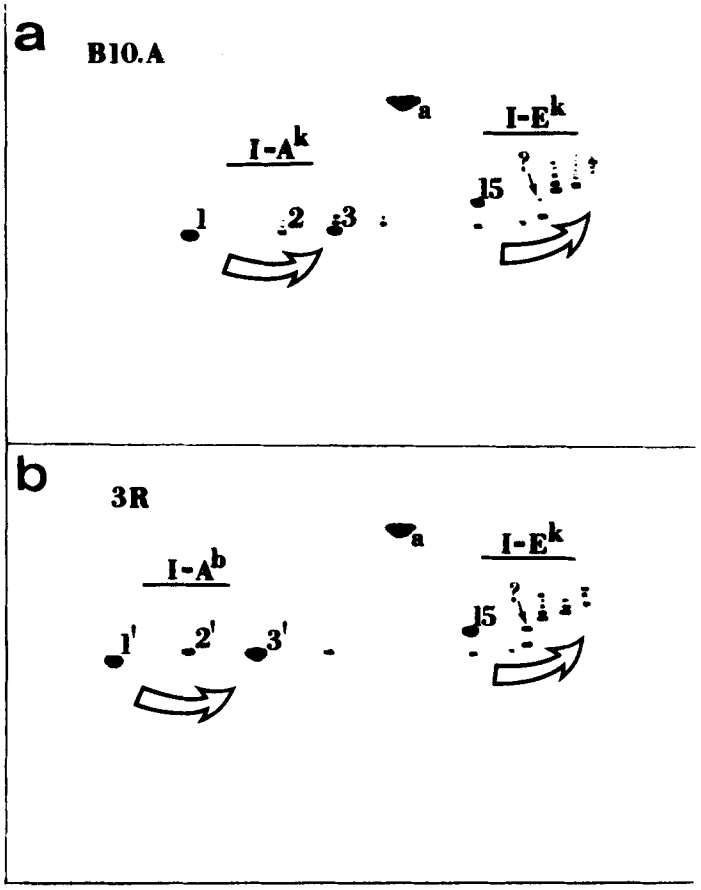

FIG. 3. Schematic showing proposed origin of molecules precipitated by anti-I-E^k antibodies. These schematized gel patterns correspond to IEF 2-D gel patterns of I-E^k precipitate obtained from a B10.A (see Fig. 2a) and b 3R (see Fig. 2d). As described in the text, spots 1, 2, 3, and the spots near them represent modified forms of an I-A^k polypeptide chain, spots 1′, 2′, and 3′ represent modified forms of an I-A^b polypeptide chain. Spot 15 and the spots near it are coded for by the I-E subregion. The spot labeled with a question mark is described later in the text. Actin is labeled with the letter a. The large arrows indicate the direction of increasing modification of the polypeptide chains.

This interpretation of the gel patterns is schematized in Fig. 3. While this model is consistent with all of the observations, direct evidence that spots 1, 2, and 3 or 1′, 2′, and 3′ represent different modified forms of the same gene product will have to be obtained by peptide mapping or amino acid sequencing techniques.

The modification of an *I-A* subregion product and its coprecipitation with *I-E* molecules appear not to be artifacts of the extraction and immunoprecipitation procedures. Anti-I-E immunoprecipitates prepared from NP-40 extracts of mixtures of [^{35}S]methionine-labeled B10 and B10.A cells produce gel patterns that resemble the pattern of B10.A alone. Sometimes a faint trace of spot 1′ from B10 is present, but the modified spots 2′ and 3′ have never been seen. Similarly, the anti-I-E precipitate obtained from an extract prepared from a mixture of 3R and 4R cells gave only the 3R pattern; spot 1 from 4R was not brought down (P. Jones, unpublished observations).

Both the specific modification and the co-precipitation of these I-A molecules may be functions of the protein coded for by *I-E*. Serological analyses have shown that the *b* haplotype, along with the *s, f,* and *q* haplotypes, do not have Ia antigenic determinants which map to *I-E* or *I-C* (22); in addition, preliminary 2-D gels (P. Jones, unpublished observations) of total cell extracts from *b, s, f,* and *q* haplotype strains have failed to reveal spots similar to I-E^k spot 15 and its modified forms which are visible in B10.A and 3R extracts (Figs. 2b and 2e). The theory that an expressed I-E antigen allows both the expression and immunoprecipitation of these I-A molecules is supported by gel analyses of I-E precipitates obtained from $(b \times d)F_1$, $(b \times p)F_1$ and $(b \times r)F_1$ heterozygotes. As with *k* haplotype strains, *d, r,* and *p* haplotype strains all have a locus or loci in *I-E* coding for Ia antigens (24). The I-E precipitates prepared from these heterozygotes generate patterns equivalent to the combination of the I-E pattern of the *d, p,* or *r* haplotype plus spots 1′, 2′, and 3′ of the 3R-like pattern (P. Jones, unpublished observations). Thus, the *d, p,* and *r* haplotypes are capable of modifying the I-A^b cytoplasmic molecule 1′ in a manner indistinguishable from that of the *k* haplotype. Similar analyses of other haplotypes in appropriate heterozygous combinations have suggested that there is an absolute correlation between the presence of an expressed I-E antigen and the modification and co-precipitation of the cytoplasmic I-A precursor.

Although the functional properties of Ia antigens are still a mystery, several mechanisms could explain how the I-E antigen could modify and allow the co-precipitation of the I-A molecules. The I-E protein itself might not modify the I-A molecules, but the binding of I-A to I-E in the cytoplasm might be essential for modification and subsequent expression on the cell surface. For example, without the I-E molecule, the I-A molecule might not have the correct conformation for insertion into the endoplasmic reticulum and/or subsequent glycosylation. Alternatively, as a glycosyl transferase the I-E protein might bind and glycosylate the I-A molecule; the two chains could then remain associated in a complex stable in NP-40.

While it seems likely that association of the I-A molecules with those coded for by the *I-E* subregion is responsible for the coprecipitation of both proteins with anti-I-E, whether the same *I-E* subregion product is responsible for the modification of the cytoplasmic I-A molecule is much less certain. Because the *k* and *d* haplotypes but not the *b* haplotype allow the expression of these I-A antigens on the cell surface, the 3R recombinant maps the locus responsible for the modification to the right of the *I-J* subregion, while the B10.A(1R) and B10.A(2R) recombinants map the locus to the left of the *H-2D* region (see Table II). Since no recombinants are available which would allow precise maping of the modification locus to *I-E*, proof that the I-E antigen itself modifies or allows the modification of the I-A protein will have to be obtained at the molecular level.

One unexplained observation concerns the spot labeled with a question mark in Figs. 1, 2, and 3. This spot is reproducibly darker on 3R, $(B10 \times B10.A)F_1$ and $(3R \times 4R)F_1$ precipitates than it is in B10.A or $(4R \times B10.A)F_1$ precipitates (see Fig. 1), indicating that a high level of expression is associated with *I-A^b*. This molecule might be an additional modified form of spot 1′, or it might represent another *I-A^b* gene product whose precipitation also requires the expression of an I-E antigen. Unfortunately, there are too many Ia spots in this region of the gels to identify subsets which might represent the products of different loci.

The small but definite differences in electrophoretic mobilities between the *I-E*

dependent I-A molecules of *b* and *k* haplotypes (called A_e^b and A_e^k, respectively) suggest that these proteins might have haplotype-specific antigenic determinants which potentially could be recognized by antisera. However, no anti-Ia^b or anti-Ia^k antisera used precipitate spot 1 from 4R or spot 1′ from B10, even though they do precipitate the products of two other *I-A* loci. These chains, called A_a and A_b, have distinctly different electrophoretic mobilities from the ones shown in the autoradiograms presented here (16).[2]

It is possible that the polymorphic differences in the A_e molecules are only weakly immunogenic. However, the detection of new Ia antigenic determinants on heterozygotes has been reported in a different system. Examining secondary mixed lymphocyte reactions in a purely in vitro system, Fathman has found that *a* haplotype cells primed to $(b \times a)F_1$ cells respond better to a second exposure of F_1 cells than they do to *b* haplotype cells, suggesting the existence of antigenic determinants unique to the heterozygote (25). These findings could be due to the kind of phenomenon reported here. Experiments aimed at detecting antibody activity towards the A_e molecules are currently in progress.

The regulation of cell surface expression of an *I-A* subregion product by a locus mapping in *I-E* (or elsewhere in the region between *I-J* and *H-2D*) has exciting implications for two-gene control of immune responsiveness or immune suppression. For a variety of antigens under *Ir* gene control, the ability to generate specific antibody responses is regulated by two loci, one (Ir_β) mapping in *I-A* or *I-B* and the other (Ir_α) mapping in *I-E* or *I-C* (6). Certain combinations of alleles at these two loci can complement each other to permit a response to occur. Similar two-gene control has also been observed for the generation of immune suppression to the synthetic polypeptide poly L-glutamic acid, L-tyrosine (6). The mechanism(s) and cellular site(s) of action of *Ir* and *Is* gene function are not known (10, 11). However, because of the antigen specificity of such systems, it is likely that they reflect recognition processes occurring at the surfaces of immunocompetent cells; indeed several types of evidence suggest that the Ia antigens are the products of the *Ir* genes (26, 27).

The regulation of expression of cell surface antigens coded for by the *I-A* subregion by a locus mapping between *I-J* and *H-2D* suggests one mechanism by which two complementing genes might control immune responsiveness. As is true for *Ir* and *Is* genes, complementation allowing I-A antigen expression can occur in either the *cis* or *trans* position. Combining the *b* and *k* or *d* haplotypes allows the expression of the A_e^b:E complex on the cell surface; this molecular structure is not found on cells of either parental haplotype. Functional capabilities unique to this complex of I-A and I-E polypeptide chains also would not be shared by either parental haplotype. In this context, it is interesting to note that in a number of complementing *Ir* and *Is* gene systems, there is a good correlation between expression of Ia antigens coded for by *I-E* or *I-C* and the presence of a complementing *Ir* or *Is* gene on the right side of the *I* region (6). The Ir_α gene thus might regulate the expression of the Ir_β gene product (the A_e protein?) on the cell surface, perhaps by coding for the I-E antigen itself.

The formation of complexes of A_e and E, demonstrated by the coprecipitation of A_e with I-E molecules from *k, d, p,* or *r* haplotypes, suggests an additional way in which two *I* region genes might complement each other to generate a unique structure not shared by either parental haplotype. Cells from a mouse heterozygous at loci in both *I-A* and *I-E*, such as a $(k \times d)F_1$ heterozygote, should express four types of A_e:E complexes: A^k:E^k, A^k:E^d, A^d:E^k; A^d:E^d. Conceivably, only some combinations of

chains from the two haplotypes might provide a structure which is stable. Furthermore, it is possible that the complexes formed from some combinations of alleles, though stable, might not be functional; in this case, no *Ir* gene complementation would be observed.

The possibility that the molecular complementation for Ia antigen expression is the basis for complementation between two *Ir* or *Is* genes raises interesting questions about the cellular site(s) of action of those *I* region genes. Cells of the B, T, and macrophage lineages have all been implicated in the genetic control of immune responsiveness, but the identity of the cells in which two-gene complementation for responsiveness occurs is not yet known. While all three cell types express Ia antigens, it is likely that the Ia antigens described in this paper are B-cell products (16) (a minor contribution from macrophages cannot be ruled out). If *Ir*-gene complementation results from Ia antigen complementation, then two-gene control of immune responsiveness may be a B-cell function. However, T cells and macrophages may also share this functional trait; whether or not complementation for Ia antigen expression also occurs in these cell types may provide a clue.

The two types of modulation of the structure of cell surface Ia antigens discussed here, regulation of expression of I-A antigens by another (possibly *I-E*) locus, and combinatorial association of chains of different subregions and haplotypes, clearly contribute additional levels of structural complexity to Ia antigens beyond that due only to genetic polymorphism. Demonstrating the roles that these phenomena play in the genetic control of immune responsiveness will require additional studies at the genetic, biochemical, and cellular levels.

Summary

Two dimensional polyacrylamide gel electrophoresis of Non-Idet P-40 extracts and of specific Ia immunoprecipitates from [^{35}S]methionine-labeled mouse spleen lymphocytes has revealed that the cell surface expression of some Ia antigens appears to be controlled by two genes. One locus, which maps in the *I-A* subregion, is probably the structural gene for an Ia polypeptide chain. The second locus, which maps between the *I-J* and *H-2D* regions, controls whether this *I-A* encoded molecule (A_e) remains in the cytoplasm or is modified and expressed on the cell surface. Complementation between these two loci allowing surface expression of A_e can occur in the *cis* or *trans* chromosomal position.

Both the I-A molecule and a polypeptide chain coded for by a locus in *I-E* are coprecipitated by anti-I-E antibodies, suggesting that these two chains are associated with each other as a multisubunit complex in the cell. Because the ability to complement *I-A* for A_e expression is a property only of those strains which synthesize an *I-E*-encoded protein, it is likely that the I-E product itself is regulating the expression of A_e.

These observations suggest several mechanisms by which interaction between two *I* region loci can generate new cell surface molecules. As a result, they may have important implications for understanding the molecular basis of two gene control of immune responsiveness and immune suppression.

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