Organization of the Immune Response Genes

Both subunits of murine I-A and I-E/C molecules are encoded within the *I* region.

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Summary. The *I* region of the major histocompatibility complex contains immune response genes that display considerable polymorphism; that is, there are many alleles at each locus. These genes regulate the immune response to antigen by mediating intercellular communication among lymphoreticular cells. An analysis of the primary structure of the products of two subregions of *I* (*I-A, I-E/C*) was undertaken in order to understand the genetic organization of the region, the evolution of the genes and, eventually, their function.

The *I* region of the major histocompatibility complex (MHC) regulates immune responsiveness [immune response (*Ir*) genes], susceptibility to viral oncogenesis, and susceptibility to particular diseases that may have an immunological pathogenesis (*1*). Thus, *Ir* genes can determine whether a mouse strain develops a strong or weak immune response to a given antigen (*2*) or develops leukemia after injection of Friend virus (*3*). In man, diseases such as rheumatoid arthritis (*4*), diabetes mellitus (*5*), and myasthenia gravis (an autoimmune disease affecting neuromuscular transmission) (*6*) are associated with particular alleles of the *HLA-D* region, which is analogous to the murine *I* region (*7, 8*). In the mouse, the gene products thus far demonstrated for the *I* region are cell surface glycoproteins, designated as the immune associated (Ia) antigens (*9*). With the use of antiserums against *I* region products, the Ia antigens have been detected principally on T lymphocytes (*10*), B lymphocytes (*11*), and macrophages (*12*). Numerous studies have demonstrated that antiserums to Ia specifically inhibit many immune phenomena, controlled by the *I* region including the induction of T cell proliferation (*13*), antibody synthesis to certain antigens that are under *Ir* gene control (*14*), successful collaboration among immunocompetent cells (*15*) and the induction of the mixed lymphocyte reaction (MLR) (*16*). These observations and the failure to find an *Ir* gene product different from Ia antigens have led to the tentative conclusion that Ia antigens are products of *Ir* genes and function as recognition units that mediate interactions among cells. Therefore, the elucidation of the structure and function of this family of molecules involved in intercellular communication is of considerable interest.

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Functional, serological, and biochemical analyses have discriminated five *I* subregions, *I-A*, *-B*, *-J*, *-E*, and *-C* (Fig. 1) (*17*). Each subregion controls phenotypic traits concerning the regulation of the immune response to particular antigens. At present, only the *I-A* (*A*) and *I-E/C* (*E/C*) subregions control biochemically defined molecules (*18*). There is extensive serological polymorphism among the *A* subregion alleles (alleles of *I* are also referred to as haplotypes) and some polymorphism at the *E/C* subregion (*19*). There is evidence that the gene products of these two subregions are expressed on the same B cell and macrophage (*20*) and that both products play critical roles in cell interaction (*21*). One current hypothesis is that A and E/C molecules interact with exogenous antigen associated with macrophages and B lymphocytes and thereby present an immunogenic complex to T lymphocytes. This results in the stimulation of T cells, which in turn are essential in the triggering of B cells to replicate and differentiate into antibody-secreting cells.

In this article, we describe the results of our primary structural analysis of the Ia alloantigens with the objectives of elucidating the genetic organization of the respective loci, their evolution, and, eventually, the molecular mechanisms through which these putative receptor molecules function.

Because of the paucity of Ia molecules on normal lymphoid tissues and the unavailability of murine cell lines producing these antigens in sufficient amounts, it has been necessary to use radioisotopic labeling techniques to examine their primary structure. In all of our studies, Ia antigens were isolated from labeled (^{3}H, ^{14}C, or ^{125}I) splenic lysates by chromatography on lentil lectin Sepharose, immunoprecipitation with alloantiserums produced in congenic strains of mice, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*22*). Both the A and E/C antigens contain two polypeptide subunits, designated alpha (α) and beta (β). We have examined the α and β subunits for primary structural variation by NH_2-terminal sequence analysis (*23–25*) and comparative tryptic peptide mapping (*26, 27*).

A Antigens Are Structually Different from E/C Antigens

We have compared the apparent molecular weight, the tendency to form disulfide-bonded α-β dimers during detergent lysis, and the primary structure of the A and E/C subunits. Using sensitive double label (^{3}H and ^{14}C) techniques (*22*), we examined the A antigens from three haplotypes for electrophoretic variation on SDS-PAGE. Under reducing conditions, the apparent molecular weights of the α and β subunits for all three of these products are 34,000 and 26,000, respectively. Under nonreducing conditions, the α and β chains are disulfide-bonded; however, lysis of the cells in the presence of an alkylating agent results in the disappearance of the α-β dimer and the appearance of only α and β chains, indicating that the disulfide bonding occurs during lysis with detergent

(*22, 28*). In contrast, two alleles (*d* and *p*) of the *E/C* subregion encode α and β subunits that have apparent molecular weights of 31,000 and 29,000, respectively; two other *E/C* alleles (*k* and *r*) have α and β subunits of 34,000 and 28,000, respectively. The E/C alloantigens do not form disulfide-bonded dimers under normal detergent lysing conditions. Hence, the A and E/C alloantigens can be distinguished by the apparent molecular weight of their subunits and by the tendency of the α and β subunits to form disulfide-bonded α-β dimers.

The allele-associated variations in molecular weight of the E/C α and β subunits was unexpected. Additional experiments (*22*) suggest that posttranslational modification, such as differences in the size or number of carbohydrate moieties, is not the cause of this variation in apparent molecular weight. These variations could result from (i) differences in the sizes of the polypeptide chains (no precedent for this in allelic products), (ii) conformational differences that might affect electrophoretic mobility, or (iii) encodement by two separate loci. That is, the differences might not be allelic in origin. Rather, the *E/C* subregion might consist of two separate but related loci in which the *k* and *r* products are encoded by one locus while *p* and *d* are encoded by a second locus. There is no information at present to exclude any of these possibilities.

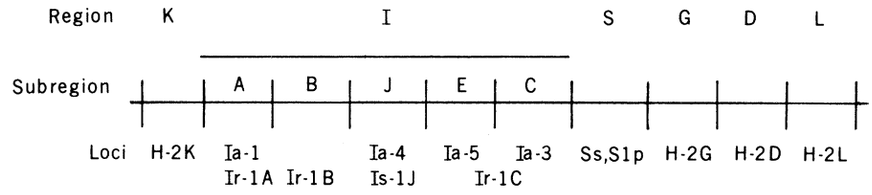

Fig. 1. Genetic map of H-2 complex. Vertical lines indicate crossovers that separate the loci.

As can be seen by comparing the data in Tables 1 and 2, analysis of the NH_2-terminal sequence reveals no apparent homology between the α chains of A and E/C or between the β chains of A and E/C. This lack of homology is supported by tryptic peptide maps of the A and E/C products (*29, 30*).

Both A and E/C Antigens Show Allele-Associated Variation

Multiple differences in the amino acid sequence between the β chains encoded by alleles of A and E/C have been observed. Table 1 shows a comparison between products of two *A* alleles. Of 12 assignments made, there are four differences between the β chains encoded by *k* and *b*. Other positions are apparently occupied by the amino acids that cannot be adequately incorporated into normal splenocytes. As yet, no differences have been noted between the α chains of *k* and *b* alleles. McMillan *et al.* (*31*) have obtained results entirely consistent with ours.

The NH_2-terminal sequences of the α and β subunits of two alleles of E/C are shown in Table 2. No differences were detected between the polypeptides of the α subunits. However, the β subunits encoded by the *r* and *k* alleles are different at positions 12 and 13. Similar results have been described for the E/C^k and E/C^d antigens by McMillan *et al.* (*31*) and Allison *et al.* (*32*).

Several *A* alleles have also been compared by tryptic peptide mapping, a means of probing the structural relatedness among proteins. This technique, however, tends to provide an overestimate of the degree of structural variation between molecules. For example, a single amino acid substitution in a peptide could so alter it that the peptide elutes from the ion-exchange column at a different position. Generally, a 40 to 50 percent homology by peptide mapping techniques represents an 80 to 90 percent homology by amino acid sequence. In

Table 1. Partial NH_2-terminal amino acid sequences of I-A alloantigens. Dashes at positions 9, 12, 13, and 14 of the β chain polypeptides indicate sequence differences between A^k and A^b. These allele-associated differences are also enclosed in a box.

Allo-antigen	Residues* at positions:																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
α chains																				
A^b				Ile		Ala			Val			Tyr				Val	Tyr			
A^k				Ile		Ala			Val			Tyr				Val	Tyr			
β chains																				
A^b			Ser		Arg	His	Phe	Val	Tyr		Phe	—	—	—		Tyr	Phe			
A^k			Ser		Arg	His	Phe	Val	—		Phe	Pro	Pro	Phe		Tyr	Phe			

*Abbreviations for the amino acid residues are Ala, alanine; Arg, arginine; His, histidine; Ile, isoleucine; Phe, phenylalanine; Pro, proline; Ser, serine; Tyr, tryosine; and Val, valine; (and in Table 2) Leu, leucine; Lys, lysine; Thr, threonine.

Table 2. Partial NH_2-terminal amino acid sequences of I-E/C alloantigens. The asterisks (*) at positions 6, 10, and 16 of the E/C^r α polypeptide indicate that Thr, Ala, and Pro were not tested. The dash at position 12 of the E/C^k β polypeptide indicates that Ser is not present. Allele-associated differences between the β polypeptides of E/C^k and E/C^r are enclosed in a box.

Allo-antigen	Residues at positions:																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
α chains																						
E/C^k	Ile	Lys			His	Thr	Ile	Ile		Ala		Phe	Tyr	Leu	Leu	Pro			Arg			Phe
E/C^r	Ile	Lys			His	*	Ile	Ile		*		Phe	Tyr	Leu	Leu	*			Arg			Phe
β chains																						
E/C^k	Val	Arg		Ser	Arg	Pro		Phe	Leu		Tyr	—	Lys	Ser				Phe	Tyr			
E/C^r	Val	Arg		Ser	Arg	Pro		Phe	Leu		Tyr	Ser	Thr	Ser				Phe	Tyr			

Table 3. Summary of peptide mapping data on the E/C antigens from recombinant strains. The arrows indicate that the β chain must be encoded to the right (→) or left (←) of that locus. N.D., not determined.

Strains	H-2 Alleles						Coelution of tryptic peptides with B10.A E/C subunits (%)	
	K	I						
		A	B	J	E	C	α	β
B10.A	k	k	k	k	k	d	100	100
B10.AQR	q→	k	k	k	k	d	N.D.	100
B10.A(5R)	b	b	b	←k	k	d	100	60
B10.A(3R)	b	b	b	b	k→	d	100	60
B10.HTT	s	s	s	s	←k	k	100	56

contrast to NH_2-terminal sequencing, peptide mapping has demonstrated structural variation between allelic products for both the α and β polypeptides. An example is shown in Fig. 2A where only 55 percent of their A^k and A^b α-subunit tryptic peptides coelute. The β chains (Fig. 2B) also show considerable allelic variation (40 percent coelution of tryptic peptides). Comparison of A^k and A^d alloantigens indicates that a similar degree of variation exists in the α and β subunits of these allelic products.

Four E/C allelic products have been analyzed by comparative peptide mapping (*26*). Small but reproducible haplotype-associated variation was detected in the α chains—the α chains of *p*, *d*, and *r* differed from that of *k* by one to three peptides (83 to 97 percent coelution). Comparisons of the β chains from the above E/C alleles revealed extensive variation, comparable to that observed for the *A* subregion α and β subunits. Only 48 to 69 percent coincident elution of peptides was found. Others (*33*), using different techniques to resolve tryptic peptides, have reported similar findings in comparing the *k* and *d* products.

In all the above studies, congenic strains of mice were used, having the same "background" genes and differing only at their MHC. Thus, the finding of allele-associated structural differences allows the conclusion that both subunits of A and both subunits of E/C are encoded by or controlled by the MHC.

The simplest interpretation is that these genes in the MHC are encoding the *A* and *E/C* gene products. An alternative possibility is that the genes that we have mapped to the MHC are in fact regulatory genes that control the activation of structural genes that reside outside the MHC. To explore this possibility, we compared peptide maps of *A* encoded α and β chains of two congenic strains (A.TL and B10.A) that have different "background" genes and the same *A* allele. No differences were noted between the products of the two strains. This result together with those of others (*34*) make it unlikely that a regulatory gene in the MHC controls a single polymorphic structural gene outside the MHC. The data, however, do not exclude a more complex possibility previously suggested by Bodmer (*35*) to explain polymorphism of the major transplantation antigens. Applying his argument to an *I* locus, each mouse strain would have a bank of tandem genes outside the MHC that is responsible for the polymorphism of Ia antigens; a regulatory gene within the particular *I* locus would activate one of these structural genes in each mouse strain. This alternative can only be definitively tested by sequencing the relevant DNA or, possibly, by appropriate somatic cell hybrid techniques.

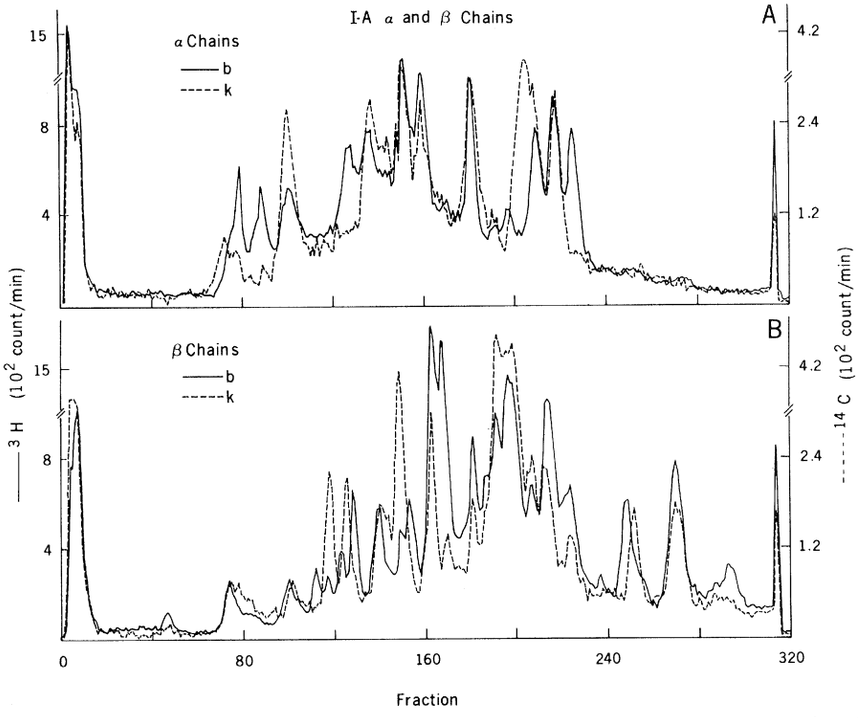

Fig. 2. Ion-exchange chromatography of tryptic digests of the α (A) and β (B) of the A^k and A^b alloantigens. ^{3}H-labeled (—) A^b α and β subunits are compared with ^{14}C-labeled (- - -) A^k α and β subunits. [Courtesy of *Journal of Immunology*]

Different *I* Subregions Encode the E/C α and β Subunits

Jones *et al.* (*34*) have demonstrated that the E/C antigens are not controlled solely by the *E/C* subregion, but by two different *I* region loci—one in *A* and the other in *E/C*. Specific immunoprecipitates obtained with antiserums to E/C were examined by two-dimensional gel analysis. The E/C alloantigens consisted of two electrophoretically distinct species of polypeptides. Further analyses were performed with F_1 hybrids and intra-*I* region recombinants. These recombinants are derived from heterozygous parents in which two chromosomes 17 have broken and rejoined in a particular position within the *I* region. The results demonstrated that one species of polypeptides was controlled by a locus in *E/C* while the other was controlled by the *A* subregion. Thus, strains with the same haplotype in *E/C* but different haplotypes in *A* expressed E/C alloantigens in which one species of polypeptides was similar whereas the second showed electrophoretic variation. It was not possible by the technique used to ascertain whether the allele-associated variations in the second species of polypeptides were due to post translational modification or to primary structural differences. In an effort to extend these observations

to α and β subunits, and to determine whether the observed electrophoretic differences were due to primary structural differences in polypeptides, we have compared E/C α and β chains from appropriate *I* region recombinants by peptide mapping (*27*).

We analyzed five strains in which the haplotype is the same in *E/C* but varies in *A* (Table 3). In such recombinants, the tryptic peptides of all α chains coeluted; however, major differences were detected among their β subunits (Fig. 3). As can be seen in Fig. 3, the E/C β chains of the 3R and 5R recombinants (*b* allele in *A*) are approximately 40 percent different from the E/C β chains of B10.A (*k* allele in *A*). Further experiments have shown that, as would be predicted, the E/C β chains from 3R and 5R are identical (both are *b* in *A*). Posttranslational modification, for example, glycosylation, could not account for such extensive structural variation and, in addition, tryptic Ia glycopeptides have been detected only in the "fall through" peptide peak from the ion-exchange column (*36*). The use of another H-2 recombinant, B10.AQR, indicates that the locus resides in *A* or *B* in confirmation of the earlier observations of Jones *et al.* (*34*). Thus, E/C β chains are encoded in *A* or *B*. Since antiserum to E/C was used for preparing all the above immunoprecipitates, and since the β subunit of E/C is encoded in *A*, it follows that the α chain must be encoded by *E/C*.

We have also compared the A alloantigens from several intra *I*-region recombinant strains that have identical alleles (*k*) in *A* but that differ on either side of *A*, that is, in *I-B* and *H-2K*. The results of peptide mapping of the α and β subunits from these recombinants indicate that both subunits of the A alloantigen are encoded by the *A* subregion.

A model of our concept of the genetic organization of the loci encoding the A and E/C alloantigens is shown in Fig. 4. For simplicity, the loci encoding the E/C α and β subunits were placed in the *E* (instead of *E/C*) and *A* (instead of *A-B*) subregions, respectively. There is, of course, no information concerning the precise sequence of genes in *A* encoding the A α, A β, and E/C β polypeptides.

Genetic Control of E/C May Explain *Ir* Gene Complementation

In addition to providing a firm basis for understanding the biochemistry of the Ia antigens, our studies as well as those of others are beginning to bridge the gap between structure and function. There are a number of examples of antigens under *Ir* gene control which require the presence of responder alleles at two distinct *I* region loci, that is, gene complementation (*37*). One example is the response to a random polymer of glutamic acid, lysine, and phenylalanine $(Glu\text{-}Lys\text{-}Phe)_n$ which requires responder alleles at the *A* and *E/C* subregions for both the generation of antibody synthesis and proliferation of T lymphocytes (*38*). One possible molecular mechanism for this complementation is that the E/C alloantigen alone determines responsiveness to $(Glu\text{-}Lys\text{-}Phe)_n$ and that the α and β chains are essential for the responsiveness and thereby account for the observed gene complementation. This interpretation is favored because of (i) the ease of blocking T cell stimulation with antiserum to E/C (that is, antibody to the E/C α chain) and (ii) the relative difficulty in blocking with antiserum to A (the few antiserums to A that are effective at blocking might have undetected specificity to the E/C β chain) (*39*). If this interpretation is correct, it provides further evidence that Ia antigens are the products of *Ir* genes. An alternative explanation is that both the A and E/C alloantigens are required for responsiveness to $(Glu\text{-}Lys\text{-}Phe)_n$ and that the complementation reflects a requirement for two responder alloantigens.

Jones *et al.* (*34*) have also reported that, in B cells from an F_1 (heterozygotic) animal, the E/C α chain derived from one parent can combine with E/C β chains from the other parent. Thus, a heterozygous animal has the capacity to express four distinct E/C antigens rather than two. It has not yet been shown that this promiscuous binding is common among the α and β chains of A. Experi-

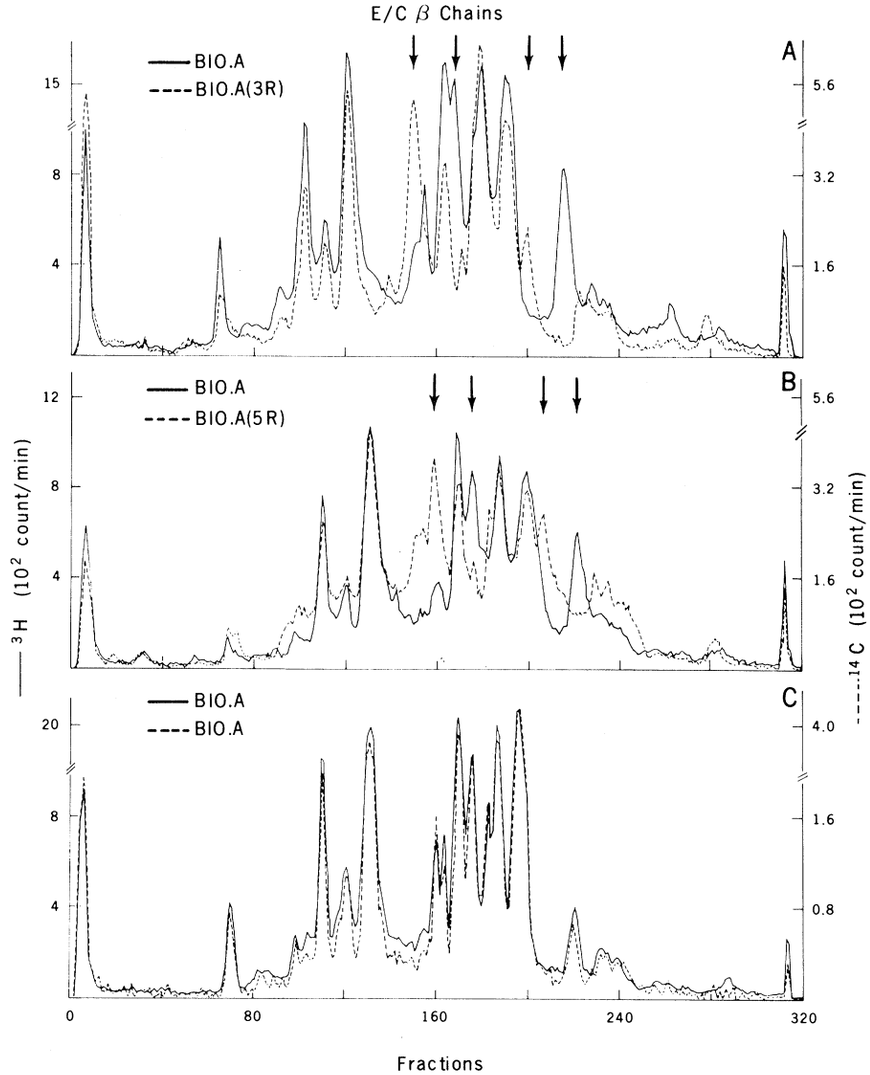

Fig. 3. Ion-exchange chromatography of tryptic digests of E/C β chain polypeptides. (—) ^{3}H-labeled E/C^k β chains from B10.A are compared with ^{14}C-labeled (- - -) E/C^k β chains from (A) B10.A(3R); (B) B10.A(5R); and (C) B10.A. Major peptide differences are denoted by arrows. [Courtesy of *Journal of Experimental Medicine*]

ments directed toward examining the A and E/C molecules from F_1 and recombinant strains should help to clarify further this potential mechanism for explaining complementation in *Ir* genes.

Murine E/C and Human I-Like Antigens (DR) Are Homologous

The limited sequence data available at present indicate a striking homology between the human DR antigens and the murine E/C alloantigens. Thus, 6 of 7 NH_2-terminal assignments are identical for the α chains and 7 of 14 for the β chains (*32, 40*). The human β chain sequences have to be shifted to the right by one amino acide residue to reveal the homology with the murine E/C β chains. No homology was evident between DR antigens and murine A alloantigens.

There is controversy concerning which subunits of the human alloantigens are MHC encoded. Two reports (*41*) indicate that the α polypeptides are encoded within the MHC, whereas another report (*42*) suggests that only the β subunit is encoded by the MHC. Because of the sequence homology between the human and murine E/C antigens, and the evidence that both E/C subunits are encoded by the *I* region, it is likely that both the α and β subunits of the human DR antigens are controlled by *HLA*-linked loci. Indeed, if the human *I* subregions are organized in general like those of the mouse, a human analog of *A* would be predicted. Additional DR specificities that could be encoded by a region distinct from *HLA-D* have been reported (*42*).

Conclusions and Implications

The conclusion that linked genes control both subunits of the A and E/C antigens (Fig. 4) is apparently the first example of such genetic control of heteropolymers except for molecules synthesized as "polyproteins" (that is, synthesized as a single polypeptide chain and subsequently cleaved enzymatically to yield two or more polypeptide chains). An example of multichained molecule (three subunits) which is MHC-linked and synthesized as a polyprotein is the complement protein C4 (*43*); the murine Ss protein (encoded to the right of *I*) is functionally and structurally analogous to human C4 (*44*). There is no evidence for or against the synthesis of Ia antigens as polyproteins, which are then cleaved to yield α and β subunits. Other products of chromosome 17—such as H-2D, H-2K, thymus leukemia (TL), and Qa antigen—each have β_2-microglobulin as a second subunit; the gene encoding β_2-microglobulin is not linked to the murine MHC (*45*). Additional examples of heteropolymers controlled by unlinked genes include immunoglobulin, hemoglobin, and lactate dehydrogenase. Apparently, there are mechanisms for coordinate regulation of products of unlinked genes. If the A and E/C antigens are not synthesized as polyproteins, what then is the selective force which keep the genes encoding the α and β subunits of these molecules linked?

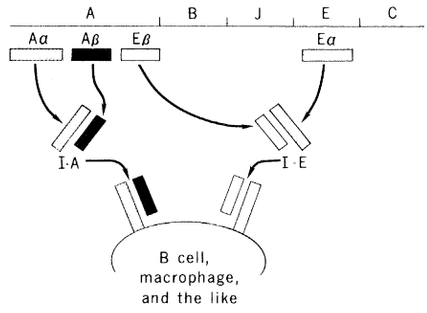

Fig. 4. Model of genetic organization and expression of the A and E/C alloantigens. The order of A α, A β, and E β "genes" in the *A* subregion is not known; also, our results have not eliminated the *B* subregion as the locus which encodes E β. For simplicity, E is used instead of E/C.

The extent of the *Ir* gene repertoire as determined by operative *I* gene products is critical to host survival because it determines the capacity of the host to mount an effective immune response to microbial invasion. At least two features of Ia antigens might favor linkage of the genes encoding the α and β subunits: (i) the extensive structural polymorphism that increases the antigen recognition repertoire of these putative receptor molecules and (ii) the requirement to maintain portions of the molecules necessary for effective subunit interaction. Perhaps the generation of structural variants, as well as the protection of subunit interaction sites, occurs in specialized portions of these molecules; these restrictions may require that both loci lie within the MHC. If the loci encoding the α and β subunits were not linked within the MHC, then unacceptable mutations or recombinations might more readily occur and thus lead to Ia molecules that are not functional. We do not yet know whether molecular constraints at the protein level control the formation of α-β dimers. For the E/C antigens, the β subunits encoded by three different alleles can each combine with the α chain encoded by one allele (*27, 34*). It is not known whether this promiscuous binding occurs among the α and β subunits of A. If the formation of hybrid Ia molecules occurs, this could increase the immune recognition capacities of individuals and explain gene complementation. If mixed molecules are not advantageous to the species, then the implication would be that only certain allelic combinations create functional α-β dimers. Hence, linkage of the α and β loci would discourage recombination and thereby decrease the formation of suboptimal α-β dimers.

Our studies indicate another difference between the major transplantation antigens and the Ia antigens. The major transplantation antigens have allele-associated structural variation of their large subunit only; β_2-microglobulin, the small subunit, is invariant within a species. In contrast, there are extensive allele-associated differences among α and β chains of A and β chains of E/C and modest allelic variation among α chains of E/C. Additional differences are that most α and β chains of the two *I* subregions are glycoproteins (*46*) and both chains of the human analog span the plasma membrane (*47*). In contrast, β_2-microglobulin is a nonglycosated protein that is not directly attached to the plasma membrane, but only to the major subunit of H-2 antigen (*45*). These structural and topographical differences probably reflect the different biological functions of the two classes of molecules, that is, the *Ir* genes under consideration have a role in the presentation of antigen by macrophages to T cells and T cell–B cell collaboration whereas the major transplantation antigens participate in killer T cell recognition of target cells (*1, 45*).

Our structural studies have revealed two other findings with genetic implications: (i) The extensive amino acid differences between the products of alleles in the two *I* subregions studied and (ii) the possible clustering of amino acid differences seen in β chains encoded by different alleles. Peptide maps of the A α, A β, and E/C β chains suggest extensive (10 to 20 percent) variation in amino acid sequence between allelic products. These findings are analogous to those found for the immunoglobulin Kappa chains of the rabbit (*48*) and rat (*49*). In these complex allotypic systems, the basis for polymorphism resides in multiple amino acid substitutions rather than the more typical single substitution. Thus, the genetic organization or evolution of the above loci is more complex than that found in simple allotypes. The E/C α chains, in contrast, show modest

allelic variation; the reason and significance for the limited degree of variability in this subunit is not clear.

The preliminary NH_2-terminal sequence data also indicate that allele-associated differences among β chains encoded by *A* and *E/C* occur in positions 9, 12, 13, and 14, and in positions 12 and 13, respectively. The data are far too limited to permit any conclusions, but the possibility of discerning allele-associated variations in particular portions of the molecule is an attractive approach for determining structure-function relationships and thereby pinpointing those parts of the molecule concerned with *Ir* gene function.

These biochemical findings may eventually provide the immunologist with a molecular basis for understanding the repertoire of *Ir* genes and the biochemistry of communication between macrophages and lymphocytes and between subsets of lymphocytes. These studies have already changed present concepts of genetic organization of the *I* region, and further information should begin to elucidate its evolution. Most important, there is a large number of human diseases in which there is an association with particular *HLA-D* alleles, as mentioned above. An understanding of the molecular basis of *Ir* gene action is necessary in elucidating the pathogenesis of these diseases.

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