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Immunology

Interaction between *I* region loci influences the expression of a cell surface Ia antigen

(*H-2* gene complex/major histocompatibility complex/mouse)

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ABSTRACT **Loci clustered in the *I* region of the murine *H-2* gene complex control the capacity to generate an immune response against foreign antigens (*Ir* loci) and control differentiation antigens which appear to serve as structures used by cells to interact with and regulate one another (*Ia* loci). Both genetic and functional studies suggest that Ia antigens may be products of *Ir* loci. Recent studies have shown that interaction between closely linked *Ir* loci is required for generating immune responses to certain foreign antigens, and that interaction between *H-2*-linked loci determines the appearance of an Ia glycoprotein (the A_e chain) on lymphocyte cell surfaces. In this report, we show that one *Ia* locus regulates the quantitative expression of the product (the E_α chain) of a second *Ia* locus. This regulatory locus is dominantly expressed and exerts its effect in either the *cis* or *trans* chromosomal position. Thus, the quantitative as well as the qualitative expression of some Ia products is dependent on interaction between tightly linked loci. Our results suggest a possible molecular basis for this regulation: the synthesis and intracellular association of A_e and E_α chains may be an absolute requirement for the expression of normal levels of either polypeptide chain on the lymphocyte cell surface. The implications these findings have for *I* region control of immune responses and study of human HLA-D antigens are discussed.**

The *I* region of the murine *H-2* gene complex is a segment of chromosome composed of a cluster of loci that control a number of immunological traits (Fig. 1) (1–3). These include: control of the capacity to generate (immune response or *Ir* loci) or suppress (immune suppression or *Is* loci) the response to a number of foreign antigens; control of antigens that are selectively expressed in functionally distinct immunocompetent cells (*I* region-associated antigen or *Ia* loci); and control of antigens that elicit allograft rejection (histocompatibility or *H* loci) or stimulate in mixed lymphocyte culture (lymphocyte-activating determinant or *Lad* loci). Genetic crossovers have permitted subdivision of the *I* region into smaller segments of chromosome, designated subregions (linear order: *I-A, I-B, I-J, I-E, I-C*).

Over the last few years, it has become evident that some *H-2*-linked loci interact with one another to generate a given phenotype. For example, interaction between the *Ir-1A* locus and the *Ir-1C* locus is required for the generation of humoral immunity against certain antigens, and interaction between the *Is-2* locus and the *Is-1* locus results in suppression of the humoral response against other antigens (4). In addition, we have shown that a locus mapping in or to the right of the *I-E* subregion regulates the cell surface appearance of the *I-A* subregion-controlled A_e polypeptide chain (5). Thus, the functional or molecular expression of *I* region loci is, in some cases, dependent on gene–gene interaction.

In this report, we show that a locus mapping in the *I-A* or possibly the *I-B* or *I-J* subregion influences the quantitative expression of the *I-E* subregion-controlled E_α chain. This regulatory locus is dominantly expressed and exerts its effect in either the *cis* or *trans* chromosomal position. These studies provide further evidence for interaction between *I* region loci and show that gene–gene interaction can influence quantitative as well as qualitative expression of an Ia polypeptide chain on the lymphocyte cell surface.

METHODS AND MATERIALS

Mice. *H-2* congeneic and recombinant strains of mice used in this study are shown in Table 1.

Antisera. Anti-I-E^k antisera used in this study include (A.CA × B10.S(7R))F_1 anti-B10.HTT, B10.A(18R) anti-B10.A(5R), and (B10 × HTI)F_1 anti-B10.A(3R). Anti-Thy-1.2 antisera include (PL/J × B6.PL-Thy-1^a)F_1 anti-B6 and monoclonal AKR anti-CBA generously provided by P. Lake (University College, London).

Dye Exclusion Microcytotoxic Assay. Details of this technique have been published (6). Briefly, 2000 spleen cells are incubated with appropriately diluted antiserum for 15 min at 37°C, washed, and incubated with rabbit complement (1:12) for 30 min at 37°C; the cells are stained with the vital dye nigrosin, and the living and dead cells are enumerated by using an inverted microscope.

Preparation of Purified B Lymphocytes. B lymphocytes were prepared by culturing spleen cells on plastic petri dishes coated with goat anti-mouse immunoglobulin and harvesting the adherent cells (7).

Immunofluorescence. Spleen cells (2×10^6) were stained with 100 μl of anti-Ia antiserum at a dilution that yielded maximal fluorescence. A second step with fluorescein-conjugated goat anti-mouse IgG was used to visualize the Ia antibody bound to the cell surface (8). The cells were maintained at 0°C throughout the staining and analysis procedure. Quantitative fluorescence analysis was performed by using a fluorescence-activated cell sorter (FACS II, Becton Dickinson, Mountain View, CA). For each histogram, 10^4 viable cells were detected by light scattering and were analyzed for fluorescence intensity (9). Identical gain settings were used in each analysis, so that the fluorescence intensities can be directly compared.

Absorption Analysis. *In vitro* absorption analysis was performed by absorbing 50 μl of 1:30 diluted antiserum with graded numbers of spleen cells for 1 hr at room temperature. The number of cells required for complete absorption of anti-

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Abbreviations: Ir, immune response; Ia, *I* region-associated antigen; A_e, lower molecular weight polypeptide chain controlled by the *I-A* subregion which associates with the E_α chain; E_α, higher molecular weight polypeptide chain controlled by the *I-E* subregion which associates with the A_e chain.

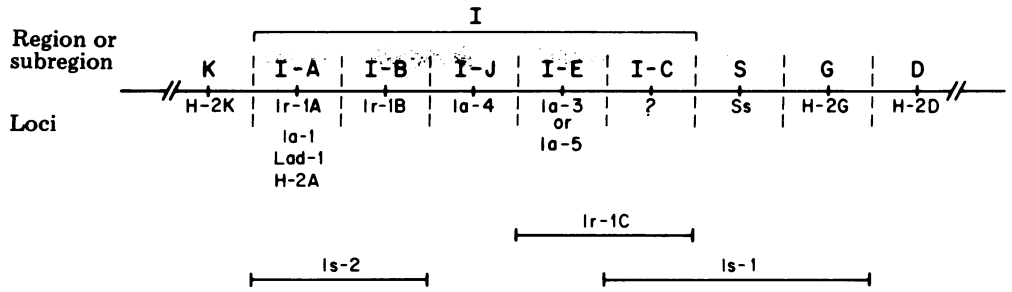

FIG. 1. Partial map of the *H-2* gene complex. Vertical broken lines indicate crossover positions that define smaller segments of chromosome (regions or subregions). The first locus listed represents the marker locus for the region or subregion. Below the marker locus are other postulated loci. Whether the marker locus for the *I-E* subregion should be designated *Ia-3* or *Ia-5* is currently unresolved. No formal designation has been proposed for the marker locus for the *I-C* subregion. H, histocompatibility; Ir, immune response, Ia, I region-associated antigen; Ss, serum substance; Lad, lymphocyte-activating determinant; Is, immune suppression. Adapted from ref. 22.

body reactive with A.TFR5 spleen cells was determined in the dye exclusion microcytotoxic assay.

Biochemical Analysis. Ia antigens were immunoprecipitated from Nonidet P-40 extracts of [^{35}S]methionine-labeled spleen cells according to published procedures (5, 10). The proteins were separated by two-dimensional polyacrylamide gel electrophoresis with nonequilibrium pH gradient electrophoresis for the charge separation in the first-dimension gels (5, 11). After the second dimension separation of $NaDodSO_4$ slab gel electrophoresis, the gels were processed for fluorography by using the autoradiographic enhancer EN^{3}HANCE (New England Nuclear). The dried gels were exposed to preflashed Kodak X-Omat R film at −70°C for 9 or 21 days.

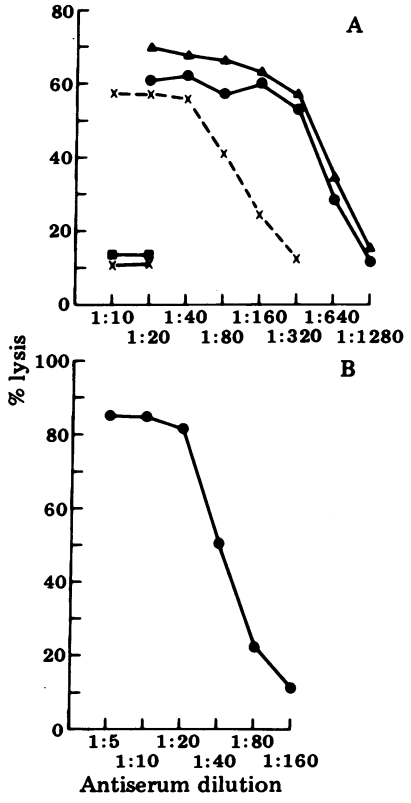

FIG. 2. Strain A.TFR5 lymphocytes express an *I-E*^{k} subregion-controlled product. [A.CA × B10.S(7R)]F_1 anti-B10.HTT antiserum analyzed in the dye-exclusion microcytotoxic assay. In all cases, treatment with complement alone resulted in 8–12% cell death. (*A*) Positive controls: A.TL (●—●) and B10.HTT (*I-E*^{k}) (▲—▲) spleen cells. Experimental: A.TFR5 (*I-E*^{k}) (x---x) spleen cells. Negative controls: A.CA (*I-E*^{f}) (■—■) and B10.S(7R) (*I-E*^{s}) (x—x) spleen cells. (*B*) A.TFR5 immunoglobulin-plate adherent splenic B cells (<2% T lymphocytes, as judged by cytolysis with anti-Thy-1.2 plus complement).

RESULTS

Recombinant Strain A.TFR5 Carries the *I-E*^{k} Subregion and Expresses an I-E^k Product. Strain A.TFR5 was derived by recombination from an (A.CA × A.TL)F_1 heterozygote (12). Our previous studies showed that a B10.A(18R) anti-B10.A(5R) antiserum, which detects *I-E*^{k} (strain A.TL) and not *I-E*^{f} (strain A.CA) subregion-controlled antigens, lysed approximately 55% of A.TFR5 lymphocytes in the presence of complement (13). These findings suggested that strain A.TFR5 derived the *I-E*^{k} subregion from progenitor strain A.TL—i.e., the crossover in strain A.TFR5 occurred inside the *I* region and to the left of the *I-E* subregion (genetic constitution *I-A*^{f} *I-B*^{p} *I-J*^{p} *I-E*^{k} *I-C*^{k}).

Data represented in this section confirm this result and show further that the majority of strain A.TFR5 B lymphocytes express an I-E^k product on the cell surface. When tested with the anti-I-E^k antiserum [(A.CA × B10.S(7R))F_1 anti-B10.HTT], roughly 55–60% of strain A.TFR5 spleen cells (Fig. 2*A*) and 85% of A.TFR5 splenic B cells (Fig. 2*B*) were killed in the presence of complement. In addition, this antiserum reacted with 55% of strain A.TFR5 spleen cells, as judged by indirect immunofluorescence analysis (Fig. 3). Finally, absorption

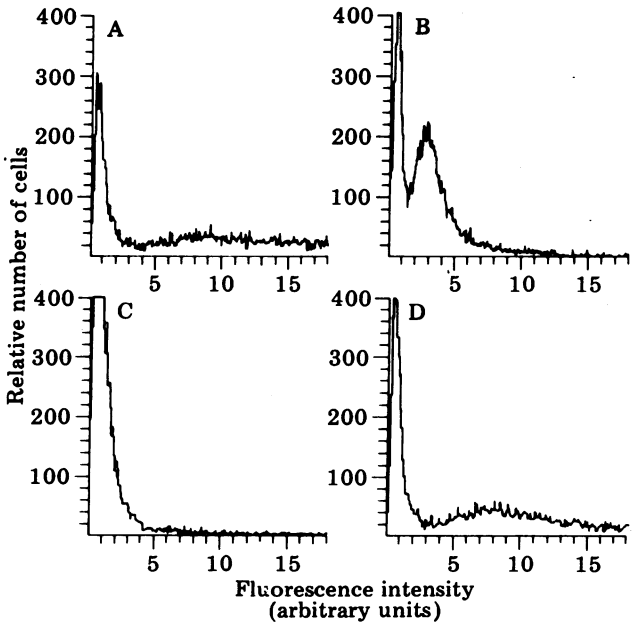

FIG. 3. Quantitative, indirect immunofluorescence analyses of spleen cells incubated with [A.CA × B10.S(7R)]F_1 anti-B10.HTT, an anti-I-E^k antiserum, as determined by analysis on a fluorescence-activated cell sorter. (*A*) A.TL (positive control, normal level of antigen); (*B*) A.TFR5 (experimental, low level of antigen); (*C*) A.CA and A.SW (negative controls, no antigen); (*D*) (A.SW × A.TFR5)F_1 (experimental, normal level of antigen).

Table 1. Absorption analysis of [A.CA × B10.S(7R)]F_1 anti-B10.HTT antiserum: Strain A.TFR5 expresses specificity Ia.7

Absorbing strain	Origin of region or subregion* I K	A	B	J	E	C	S	G	D	Cells to absorb antibody† Exp. I	Exp. II	Exp. III	Antibody absorbed	Absorbing strain expresses Ia.7*
B10	b	b	b	b	b	b	b	b	b	—	—		−	−
B10.D2	d	d	d	d	d	d	d	d	d	9	6		+	+
A.CA	f	f	f	f	f	f	f	f	f	—			−	−
B10.BR	k	k	k	k	k	k	k	k	k	9		2	+	+
B10.P	p	p	p	p	p	p	p	p	p	11			+	+
B10.G	q	q	q	q	q	q	q	q	q			—	−	−
B10.RIII	r	r	r	r	r	r	r	r	r	21	5	3	+	+
B10.S,A.SW	s	s	s	s	s	s	s	s	s			—	−	−
B10.A,A	k	k	k	k	k	d	d	d	d	10			+	+
B10.A(2R)	k	k	k	k	k	d	d	d	b	11			+	+
B10.A(4R)	k	k	b	b	b	b	b	b	b		—	—	−	−
HTI	b	b	b	b	b	b	b	?	d		—		−	−
B10.A(3R)	b	b	b	b	k	d	d	d	d	10	4		+	+
B10.A(5R)	b	b	b	k	k	d	d	d	d	9	8		+	+
B10.A(18R)	b	b	b	b	b	b	b	?	d	—			−	−
B10.S(7R)	s	s	s	s	s	s	s	s	d	—	—		−	−
B10.HTT	s	s	s	s	k	k	k	k	d	10	6	2	+	+
B10.S(9R)	s	s	?	k	k	d	d	d	d		6	6	+	+
B10.S(8R)	k	k	?	?	s	s	s	s	s	—	—		−	−
B10.AQR	q	k	k	k	k	d	d	d	d		5	4	+	+
B10.T(6R)	q	q	q	q	q	q	q	?	d	—	—		−	−
B10.P(13R)	p	p	p	p	p	?	b	b	b			3	+	+
A.TL	s	k	k	k	k	k	k	k	d	12	8	4	+	+
A.TH	s	s	s	s	s	s	s	s	d	—	—		−	−
A.TFR1	s	k	k	k	k	k	k	f	f	10		2	+	+
A.TFR5	f	f	?	?	k	k	k	k	d	80		30	+	(+)

* Adapted from ref. 22.

† Number of cells ($\times 10^{-6}$) required to completely absorb antibody reactive with A.TFR5 spleen cells. —, No absorption with ≥100 × 10^6 cells. Dye exclusion microcytotoxic assay.

studies showed that the Ia specificity detected on A.TFR5 lymphocytes was indistinguishable from the *I-E*^{k} subregion-controlled specificity Ia.7 (Table 1).

Lymphocytes from Strain A.TFR5 Express Less of the I-E^k Product on the Cell Surface Than Do Lymphocytes from Other Strains. During the above absorption analyses, we repeatedly observed that roughly 5- to 10-fold more A.TFR5 lymphocytes were required to completely absorb anti-I-E^k antibody reactive with A.TFR5 target cells than with cells from other Ia.7-positive strains (Table 1). (Preliminary reports describing this observation can be found in refs. 13 and 14.) These results suggested that less of the I-E^k product is expressed on the cell surface of A.TFR5 lymphocytes. Confirmation of this observation came from quantitative immunofluorescence analyses. When the antiserum was used at dilutions that gave maximal fluorescence, significant differences in the amounts of antigen expressed per cell were observed between strains (Fig. 3). Although the same percentages of cells were labeled, spleen cells from A.TFR5 animals expressed roughly one-fifth the amount of antigen found on spleen cells from strain A.TL. Similar results were obtained with a second anti-I-E^k antiserum [B10.A(18R) anti-B10.A(5R); data not shown].

Evidence That a Second *I* Region Locus Regulates the Quantitative Expression of the I-E^k Product on the Lymphocyte Cell Surface. Studies with A.TFR5 animals crossed to different *H-2* congeneic and recombinant strains showed that quantitative differences in the expression of the I-E^k product are regulated by a second, dominantly expressed *I* region locus. When A.TFR5 was crossed with strain B10, B10.A(4R), or B10.S(7R) (none of which absorbs anti-I-E^k antibody reactive with A.TFR5 lymphocytes), normal levels of the I-E^k product were expressed, as determined by quantitative absorption analysis (Table 2). Similarly, when A.TFR5 was crossed with strain A.SW (Fig. 3) or B10.A(4R) (data not shown), approximately 4 times more antigen was detected per cell, compared to A.TFR5, as judged by quantitative immunofluorescence. In contrast, when A.TFR5 was crossed with strain B10.T(6R), low levels of the antigen were expressed (Table 2).

Table 2. An *H-2*-linked locus mapping to the left of the crossover in A.TFR5 regulates the quantitative expression of an I-E^k antigen

Absorbing strain	Cells to absorb antibody* Exp. I	Exp. II	Exp. III	Relative amount of I-E^k antigen expressed
A.TL		6		Normal
B10.HTT	6	9	3	Normal
(7R × A.CA)F_1	—	—	—	None
A.TFR5	35	65	30	Low
(B10 × A.TFR5)F_1	12	16		Normal
(4R × A.TFR5)F_1	9	13	3	Normal
(7R × A.TFR5)F_1			3	Normal
(6R × A.TFR5)F_1	85	50		Low
(4R × A)F_1		11		Normal
(18R × A)F_1		13		Normal

* Number of cells ($\times 10^{-6}$) required to completely absorb antibody reactive with A.TFR5 spleen cells. —, No absorption with ≥100 × 10^6 cells. Dye exclusion microcytotoxic assay.

These results show that a dominantly expressed *H-2*-linked locus, mapping to the left of the crossover position in strain A.TFR5, regulates the quantitative expression of an I-E^k product on the cell surface. Alleles at this regulatory locus carried by strains B10(*b*), B10.A(4R)(*k* or *b*), and B10.S(7R) or A.SW(*s*) determine normal levels of the I-E^k product; alleles at this regulatory locus carried by strains A.TFR5(*f*) and B10.T(6R)(*q*) determine low levels of the product. The regulatory locus can act in either the *cis* (e.g., strain A.TL) or *trans* (e.g., [B10.A(4R) × A.TRF5]F_1 hybrids) chromosomal position. Combined data with recombinant strains A.TFR5 and B10.AQR (Table 1) position the regulatory locus in the *I-A* or possibly the *I-B* or *I-J* subregion (the haplotype origin of the *I-B* and *I-J* subregions carried by strain A.TFR5 remains to be determined).

Biochemical Evidence for Regulation of the Cell Surface Expression of the E$^k_\alpha$ Chain. In order to determine the molecular basis for the variable quantitative expression of the I-E^k product, immunoprecipitates were prepared from A.TL, A.TFR5, and [B10.A(4R) × A.TFR5]F_1 mice by using the anti-I-E^k antiserum (B10 × HTI)F_1 anti-B10.A(3R). In I^k strains, anti-I-E^k antisera precipitated three noncovalently associated polypeptide chains, E_α (M_r 33,000, controlled by the *I-E* subregion), A_e (M_r 28,000, controlled by the *I-A* subregion, previously referred to as E_β), and I_i (M_r 31,000, an invariant polypeptide chain found in all anti-I-region immunoprecipitates) (3, 5, 15). Fig. 4*b* shows a 9-day fluorographic exposure of the anti-I-E^k immunoprecipitate from strain A.TL. In this somewhat overexposed fluorogram, the E$^k_\alpha$, A^k_e, and I_i polypeptide chains are visible as heterogeneous arrays of spots which represent different glycosylated forms of these proteins (10). Immunoprecipitates prepared in an identical fashion from A.TFR5 are shown in Fig. 4 *c* and *d*. The I_i chain clearly is present, although in reduced intensity, but only faint spots are seen in the vicinity of the other Ia molecules (due to expression of less antigen). To obtain a better visualization of the pattern, a longer fluorographic exposure (21 days) was prepared of the

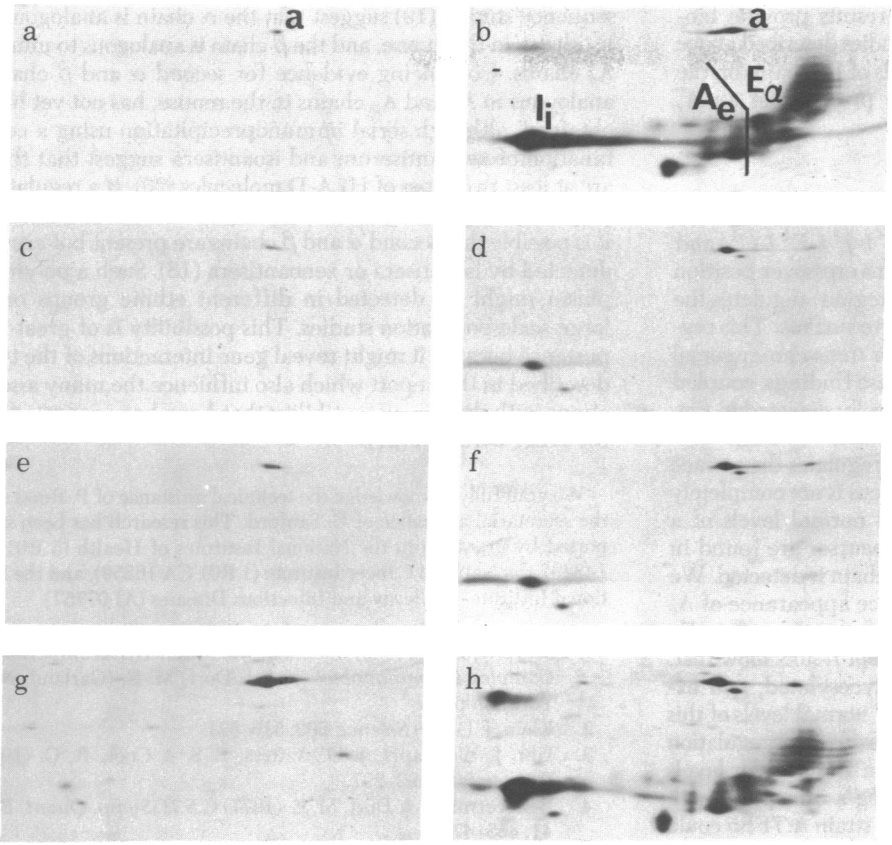

FIG. 4. Two-dimensional polyacrylamide gel electrophoresis of anti-I-E^k immunoprecipitates. Extracts from [^{35}S]methionine-labeled spleen cells from the indicated strains were immunoprecipitated with either normal mouse serum (*a*, *c*, *e*, and *g*) or anti-I-E^k antiserum [(B10 × HTI)F_1 anti-B10.A(3R)] (*b*, *d*, *f*, and *h*). Each panel represents the immunoprecipitate from an extract of 2×10^7 cells treated with 60 μl of serum. Fluorographic exposures were 9 days for *a*, *b*, *c*, *d*, *g*, and *h* and 21 days for *e* and *f*. a, position of actin in *a* and *b*. (*a* and *b*) A.TL; (*c* and *d*) A.TFR5; (*e* and *f*) A.TFR5; (*g* and *h*) [B10.A(4R) × A.TRF5]F_1.

A.TFR5 gels and is shown in Fig. 4 *e* and *f*. It is clear that the anti-I-E^k immunoprecipitate from A.TFR5 cells generates spots in the same position as the A.TL E^k_α polypeptide chain, including the most highly glycosylated forms which are on the cell surface (14) (corresponding to the most acidic of the E^k_α spots visible in Fig. 4*b*). No A^f_e chains can be detected in the A.TFR5 immunoprecipitate. These results show that the I-E^k product detected in strain A.TFR5 lymphocytes is the E^k_α chain, consistent with the observation that low levels of this chain are detected when A^f_e chains are absent.

Regulation of the levels of expression of the E^k_α chain by a second *I* region locus is verified by the anti-I-E^k immunoprecipitate prepared from an [B10.A(4R) × A.TFR5]F_1 heterozygote (Fig. 4*h*). This gel was fluorographed for the same length of time (9 days) as the corresponding A.TL gel (Fig. 4*b*). The patterns are virtually identical in their configuration, and the spot intensities are similar. Therefore, it is apparent that the [B10.A(4R) × A.TFR5]F_1 heterozygote (loci in *trans* configuration) synthesizes and processes A^k_e and E^k_α polypeptides in a fashion nearly identical qualitatively and quantitatively to that of strain A.TL (loci in *cis* configuration). Similar results were obtained with heterozygotes prepared between A.TFR5 and

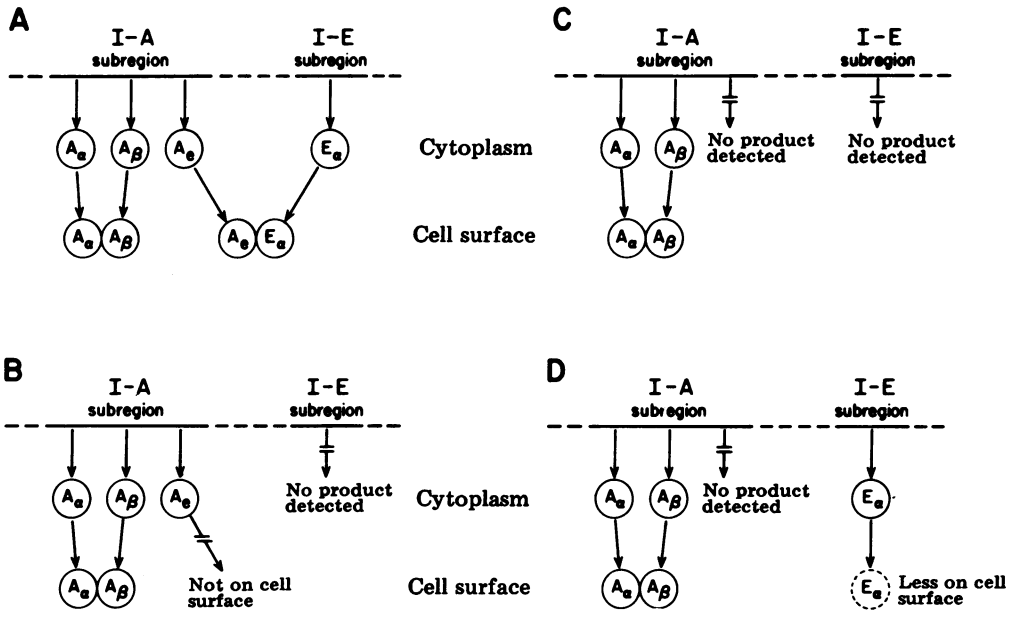

FIG. 5. Schematic interpretation of the expression of *I* region-controlled molecules. (Not all combinations of alleles were formally tested.) It is not yet clear whether the locus encoding the A_e chain or another closely linked locus regulates the expression of the E_α chain on the cell surface, and vice versa. (*A*) $I\text{-}A^{b,d,k,p,r,s}$-controlled A_e chains are found on the cell surface in strains that express $I\text{-}E^{d,k,p,r}$-controlled E_α chains. (*B*) $I\text{-}A^{b,d,k,p,r,s}$-controlled A_e chains are found in the cytoplasm but not on the cell surface in strains in which no E_α chain is detected ($I\text{-}E^{b,f,q,s}$). (*C*) No A_e chains or E_α chains are detected in strains that carry the $I\text{-}A^{f,q}$ and $I\text{-}E^{b,f,q,s}$ subregions. (*D*) Less $I\text{-}E^{d,k,p,r}$-controlled E_α chains are found on the cell surface in strains in which no A_e product is detected ($I\text{-}A^{f,q}$).

B10 or A.SW (data not shown). These results provide biochemical confirmation of the serological studies described above and reveal that expression of normal levels of E^k_α chains on the lymphocyte surface correlates with the presence of an A_e chain.

DISCUSSION

Data presented here localize the crossover in recombinant strain A.TFR5 inside the *I* region (i.e., $I\text{-}A^f\ I\text{-}B^p\ I\text{-}J^p\ I\text{-}E^k\ I\text{-}C^k$) and show that a locus mapping to the left of this crossover position (i.e., in the *I-A* or possibly *I-B* or *I-J* subregion) regulates the levels of the E^k_α chain on the B-lymphocyte surface. This regulatory locus is functional in either the *cis* or *trans* chromosomal position and is dominantly expressed. These findings, coupled with our earlier observations, are schematically depicted in Fig. 5.

The mechanism by which one *Ia* locus regulates the surface expression of the product of a second *Ia* locus is not completely understood. Previously, we showed that normal levels of a partially glycosylated A_e cytoplasmic precursor are found in lymphocytes from strains in which no E_α chain is detected. We hypothesized that regulation of the surface appearance of A_e chains required synthesis of E_α chains, formation of A_e·E_α complexes, and glycosylation (5). Our present results show that, when free E_α chains are synthesized, glycosylated, and expressed on the cell surface, the detection of normal levels of this chain is dependent on the synthesis of A_e chains. This regulation holds for both surface E_α chain expression and cellular levels of the E_α polypeptide radiolabeled during a 5-hr pulse with [^{35}S]methionine. Low levels of E^k_α chains in strain A.TFR5 could be due to either a slow rate of synthesis or a rapid rate of degradation.

The functional relevance of such gene–gene interaction is unclear. In this regard, the possible relationship between *Ia* loci and *Ir* loci is most intriguing—i.e., are Ia antigens products of *Ir* loci? For example, interaction between *Ir* loci mapping in the $I\text{-}A^b$ and $I\text{-}E^k$ subregions is required for generating an immune response to the synthetic antigen G,L,Phe (4), whereas interaction between *Ir* loci mapping in the $I\text{-}A^k$ and $I\text{-}E^k$ subregions is required for generating an immune response to pigeon cytochrome *c* (16). Responses to G,L,Phe and pigeon cytochrome *c* correlate with the surface appearance of the A^b_e and A^k_e chains, respectively, which appears to be dependent on the synthesis and expression of E_α chains.

In addition, we have recently found that monoclonal antibody reactive with A_e·E_α complexes specifically blocks T-cell proliferative responses to G,L,Phe and pigeon cytochrome *c* (unpublished data). Thus, responsiveness to these antigens correlates with the expression of certain A_e·E_α complexes and can be blocked with antibody specific for these complexes. An understanding of the genetic control of the expression of Ia antigens on lymphocyte surfaces should provide a base for unraveling mechanisms of *Ir* gene regulation.

This type of complex regulatory polymorphism in the expression of Ia antigens has important implications for our understanding of HLA-D antigens, the human analogue of the Ia antigens. Two-dimensional polyacrylamide gel electrophoresis of HLA-D antigens with a nonpolymorphic monoclonal mouse anti-HLA-D and a rabbit anti-HLA-D xenoantiserum (17, 18) reveals the presence of a single set of acidic nonpolymorphic α chains, a single set of basic polymorphic β chains, and an invariant chain analogous to the murine I_i chain. Amino acid sequence studies (19) suggest that the α chain is analogous to E_α chains in the mouse, and the β chain is analogous to murine A_e chains. Convincing evidence for second α and β chains, analogous to A_α and A_β chains in the mouse, has not yet been obtained, although serial immunoprecipitation using a combination of xenoantiserum and isoantisera suggest that there are at least two types of HLA-D molecules (20). If a regulatory polymorphism analogous to that described here exists in man, it is possible that second α and β chains are present but are not detected by isoantisera or xenoantisera (18). Such a polymorphism might be detected in different ethnic groups or in large-scale population studies. This possibility is of great importance because it might reveal gene interactions of the type described in this report which also influence the many associations with disease susceptibility that have been reported for the HLA-D region (21).

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