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Characterization of N-Type and Dually Permissive Cells Segregated from Mouse Fibroblasts Whose *Fv-1* Phenotype Could Be Modified by Another Independently Segregating Gene(s)

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Though the inbred DDD mouse strain is essentially of the N type, the primary culture of this strain was about 100-fold more sensitive to B-tropic WN1802B virus than were the typical N-type strains (C3H/He, C57L, etc.). After cloning, DDD mouse cells segregated two types of cells, typical N-type cells and cells lacking in *Fv-1* restriction. As both types of cells so far tested retained glucose-6-phosphatase-1 coded by a locus closely linked to *Fv-1* and genetic cross experiments indicated the presence of a gene(s) modifying the *Fv-1* phenotype, variation in *Fv-1* restriction could presumably be brought about by genetic changes in a gene(s) other than *Fv-1* itself. N-type and dually permissive cell clones were similarly established from the inbred G mouse. Compositions of polypeptides labeled with [^{35}S]methionine in the N-type and dually permissive cells of DDD and G mouse origins were compared by two-dimensional gel electrophoresis. The polypeptide maps of these cells were similar except for a few spots. Among these dissimilar spots, a spot of about 20,000 daltons with a pI of about 5.5 was always present in N-type cells, whereas it was absent in dually permissive cells. In DDD mouse-derived clones, a proportional relation was observed between the intensity of the spot and the restriction to the B-tropic virus.

The *Fv-1* locus regulates sensitivity of mouse cells to infection with ecotropic mouse leukemia viruses (MuLV) (5, 9). The gene is located one map unit from *Gpd-1* (a gene coding for glucose-6-phosphate dehydrogenase-1) on chromosome 4 (14). Mice with the *Fv-1*$^{n/n}$ genotype were resistant to B-tropic virus, those with *Fv-1*$^{b/b}$ were resistant to N-tropic virus, and those with *Fv-1*$^{n/b}$ were resistant to both; the resistance is a dominant trait (9). The resistance of the incompatible host to MuLV is an intracellular event (4, 8, 19). The restriction occurs at the level of circularization (7, 18) or integration of proviral DNA (6), or both. So far, identification of the *Fv-1* gene product controlling these events has scarcely been attempted except for earlier work by Tennant et al. (17), who detected inhibitor of the leukemia virus host range types in the cellular extracts. To approach this problem, we isolated N-type and dually permissive cells from two different sources and compared polypeptide compositions of these mutants.

MATERIALS AND METHODS

Cells. Embryos of 16 to 18 gestation days were obtained from C57L (*Fv-1*$^{n/n}$), C3H/He (*Fv-1*$^{n/n}$), NFS (*Fv-1*$^{n/n}$), BALB/c (*Fv-1*$^{b/b}$), and DDD (*Fv-1*$^{n/n}$) mice maintained in our institute and were used for preparation of fibroblast cultures. The medium was Eagle minimal essential medium supplemented with 7.5% fetal calf serum.

Virus. N-tropic FN-2 and B-tropic WNB-2 were obtained, respectively, from Friend leukemia virus and WN1802B virus after two successive limiting dilutions in SC-1 cells (22). The viruses were titrated by the standard UV-XC assay (13).

Estimation of *Fv-1* restriction. C57L and BALB/c secondary cultures were used as standard N- and B-type cells, respectively. Sensitivity to N- or B-tropic MuLV was expressed by the ratio of the virus titer in the cells in question to the titer in the sensitive standard cells (C57L for N-tropic virus and BALB/c for B-tropic virus). The ratio was called the N or B ratio, respectively. For estimating *Fv-1* restriction, the B ratio was divided by the N ratio to obtain the B/N ratio. B/N ratios of C57L and C3H/He were 0.002:0.01, and that of BALB/c was 100:1,000. *Fv-1* restrictions in different cell lines were compared whenever possible with the B/N ratio obtained at the same virus dilution because titration did not follow one-hit kinetics in nonpermissive cells.

Isozyme. Glucose-6-phosphate dehydrogenase-1 was analyzed by the method described by Nichols and Ruddle (10). Four confluent cultures in 100-mm petri dishes were used as a source of enzyme preparation.

Metabolic labeling of the cells and two-dimensional gel electrophoresis. Cells were labeled with [^{35}S]-methionine (50 μCi/ml, 1,050 Ci/mmol, Radiochemical Centre, Amersham, England) for 16 h. Cells were disrupted in 10 mM Tris-hydrochloride (pH 7.4)–1 mM KCl–1.5 mM $MgCl_2$–1 mM dithiothreitol with a Dounce homogenizer, and one-ninth volume of 300 mM Tris-hydrochloride (pH 7.4)–1,250 mM KCl–50 mM magnesium acetate–10 mM dithiothreitol was added immediately. After centrifugation at 2,500 rpm for 5 min, the supernatant was transferred to a test tube. The pellet was added with 1 ml of water and centrifuged at 2,500 rpm for 5 min; the water was removed immediately. The supernatant was dialyzed against water overnight. Both pellet nuclear fraction and supernatant cytosol fraction were lyophilized, dissolved in 9.5 M urea, 2% (wt/vol) Nonidet P-40, 2% ampholines (comprised of 1.6% pH range 5 to 7 and 0.4% pH range 3 to 10), and 5% β-mercaptoethanol. Polyacrylamide gel electrophoresis was performed as described by O'Farrell (11).

RESULTS

MuLV sensitivity of secondary cultures of DDD mouse cells and segregation of N-type and dually permissive cells during culture. In Fig. 1, titers of N-tropic and B-tropic viruses in C57L, BALB/c, and DDD secondary cultures are shown. DDD mouse cells, though essentially of the N type, were about 100-fold more sensitive to B-tropic virus than were C57L cells. Nine embryos from a DDD mother were individually cultured for 10 passages (about 3 months) without cloning. As shown in Fig. 2, some (cultures 3 and 7) became more sensitive to B-tropic virus than the original, whereas others remained relatively resistant (cultures 4 and 8). Cultures 3 and 8, representatives of each type, were cloned twice. From culture 3, clones which were equally sensitive to B- and N-tropic viruses (D3a, D3c, D3h1, etc.) and those with the original sensitivity (clones D3b, D3h10, etc.) were obtained. From culture 8, clones with high *Fv-1*^{n} restriction (clones D8e, D8n, D8b1, D8b10, etc.) and those with weak restriction (clones D8b, D8k, D8b6, etc.) were obtained. The situation is quite similar to that observed by Hartley and Rowe (3) in feral mouse-derived fibroblasts.

Figure 3 shows titers of N- and B-tropic viruses in typical N-type clones D8b1 and D8n and in dually permissive D3b and D3h6. In the latter clones, not only was the level of sensitivity to B-tropic virus increased, but, in addition, the

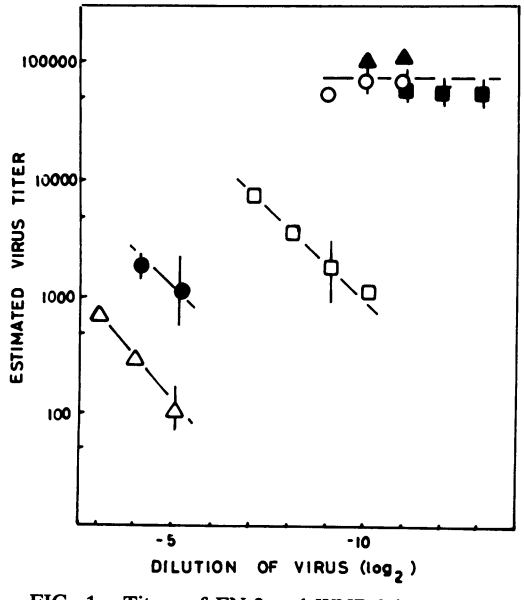

FIG. 1. Titers of FN-2 and WNB-2 in secondary cultures of C57L, DDD, and BALB/c mouse cells. Estimated virus titer is the product of the dilution factor and the number of plaques at that dilution. Symbols: △ and ▲, C57L; □ and ■, DDD; ○ and ●, BALB/c. Closed symbols indicate FN-2 titers, and open symbols indicate WNB-2 titers.

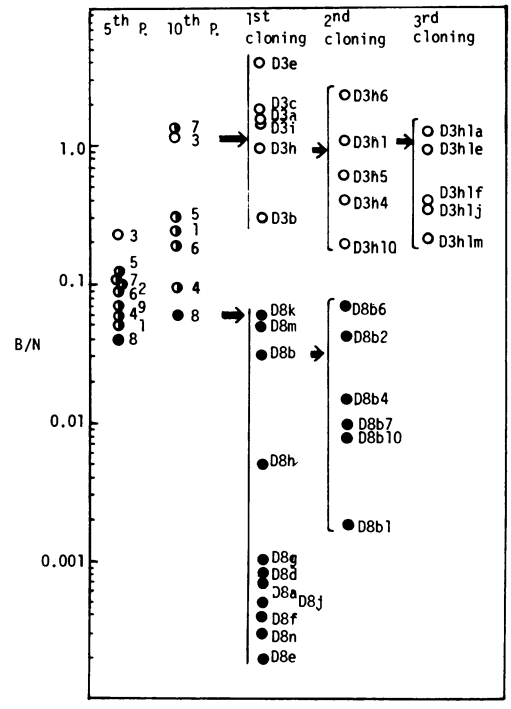

FIG. 2. *Fv-1* restriction of DDD mouse-derived clones. Nine DDD mouse embryos were cultured individually for 10 passages and then cloned successively twice. The MuLV sensitivity was examined at 5 and 10 passages. The figures 1, 2, 3, etc., are culture numbers. Culture 3 and its clones and subclones are indicated with open circles, and culture 8 and its clones and subclones, with closed circles. Half-shaded circles indicate cultures which were not cloned at all. The B/N ratio was calculated as described in Materials and Methods.

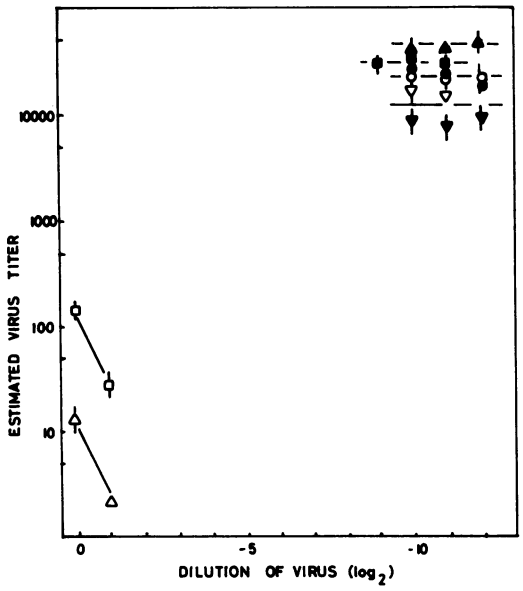

FIG. 3. Titers of FN-2 and WNB-2 in clones derived from DDD mouse embryos. Symbols: △ and ▲, D8b1; □ and ■, D8n; ○ and ●, D3b. ▽ and ▼, D3h6. Closed symbols indicate FN-2 titers, and open symbols indicate WNB-2 titers.

titration pattern, which was two hit in the restrictive host in accordance with previous reports (12, 20), turned to one hit. Typical N-type clones showed greater *Fv-1*^{n} restriction than the original uncloned cells.

As loss of *Fv-1* restriction might have been due to a deletion of all or a part of chromosome 4, including the *Fv-1* region, we examined the presence of glucose-6-phosphate dehydrogenase-1 isozyme coded by *Gpd-1*, one map unit from *Fv-1*. All the cells lacking in *Fv-1* restriction (D3h5, D3h6, D3b, D3h, etc.) as well as the typical N-type cells (D8n, D8b7, etc.) so far tested retained the isozyme.

Gene(s) modifying *Fv-1* restriction may exist. As dually permissive cells retained *Gpd-1*, which is closely linked to *Fv-1*, the loss of *Fv-1* restriction may have been due to changes in another gene(s) which could modify *Fv-1* phenotype. The weak restriction of B-tropic virus in DDD mouse cells might have been due to the presence of such a modifier gene(s). Genetic crosses were done to test this possibility. Figure 4 shows the relative sensitivities to B-tropic virus (B/N ratio) of secondary cultures obtained from individual embryos of C57L, DDD, and their F_1 and F_2 progenies. The B/N ratio of C57L ranged from 0.002 to 0.01, whereas that of DDD ranged from 0.1 to 0.5. The B/N ratio of F_1 was intermediate between those of the parents, and in F_2 the ratio was distributed widely from the range of C57L and that of DDD. To determine whether the weak restriction of DDD was a property of *Fv-1*^{n} itself or due to another gene(s)

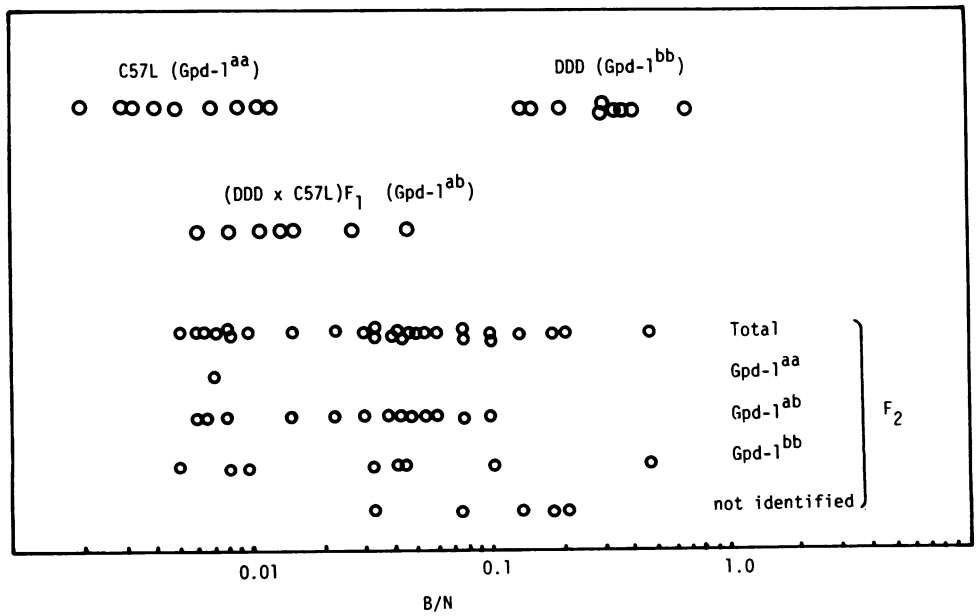

FIG. 4. B/N values of C57L, DDD, and their F_1 and F_2 progenies. A litter of C57L, DDD, and (DDD × C57L)F_1 and two litters of F_2 were trypsinized and cultured individually. The B/N ratio was plotted in the logarithmic scale. In the first line, B/N values of DDD and C57L are plotted, and in the second line, those of F_1. In the bottom of the figure, F_2 individuals were classified according to *Gpd-1* type. Each circle represents a single embryo-derived culture.

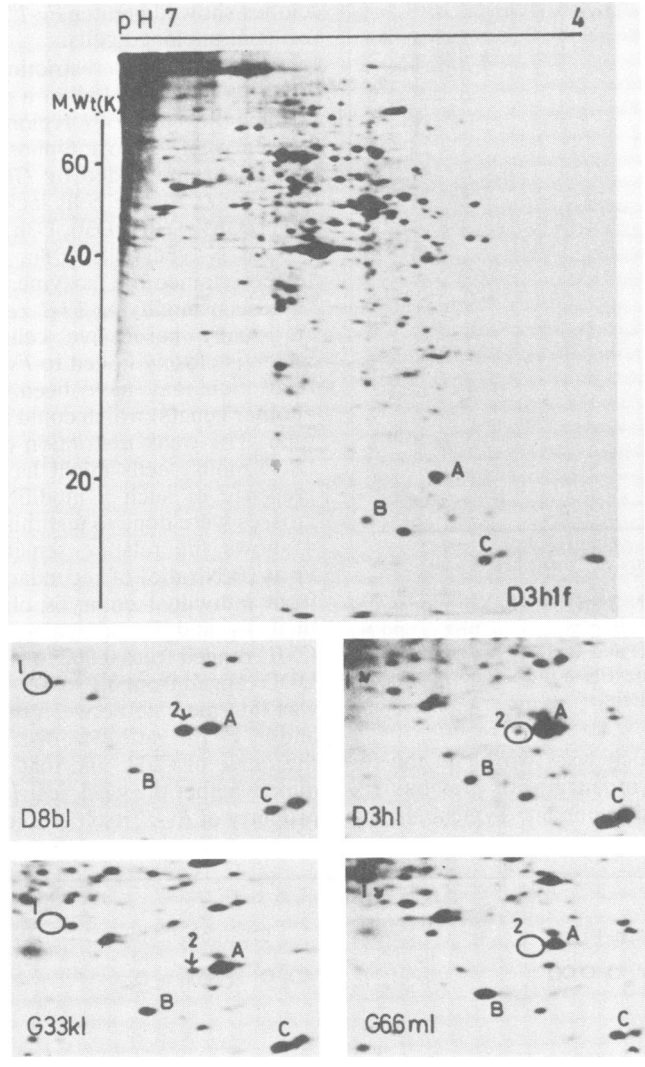

FIG. 5. Two-dimensional gel electrophoresis of nuclear fraction of DDD and G mouse-derived clones. A picture of the entire electrophoresis of D3h1f and local pictures around spot 2 of D3h1, D8b1, G66m3, and G33k1 are shown. Spots A, B, and C appeared in almost all the preparations examined and were useful for detecting spot 2. Estimations of molecular weight and pI were made with the gels run in parallel.

which modified expression of *Fv-1*^{n}, we examined the linkage between *Gpd-1* and the B/N ratio in F_2. C57L is *Gpd-1*$^{a/a}$ and DDD is *Gpd-1*$^{b/b}$. The data are shown at the bottom of Fig. 4. The segregation ratio of the *Gpd-1* genotype, a/a:a/b:b/b, was 1:13:8. The ratio was compatible with a 1:2:1 Mendelian segregation ratio (chi square = 5.8, P = 0.05 to 0.1). The B/N ratio of *Gpd-1*$^{b/b}$ individuals ranged from the values of C57L to those of DDD. Therefore, the gene(s) regulating the B/N ratio was not linked to *Gpd-1* or, consequently, to *Fv-1*.

Two-dimensional gel analysis of DDD and G mouse-derived clones. We obtained N-type and dually permissive cells from two different sources, DDD (this report) and G mice (21). If a common difference was observed in the same polypeptide in the both pairs, such a polypeptide could be a candidate for the protein involved in *Fv-1* restriction. Dually permissive D3h1 and N-type D8b1 of DDD mouse origin and dually permissive G66m1 and N-type G33k1 of G mouse origin were analyzed for [^{35}S]methionine-labeled polypeptides. Though overall electrophoretic patterns were quite similar, within each pair there were differences in at least six spots, i.e., some were present in one cell type but not in the other. Among these dissimilar spots, a

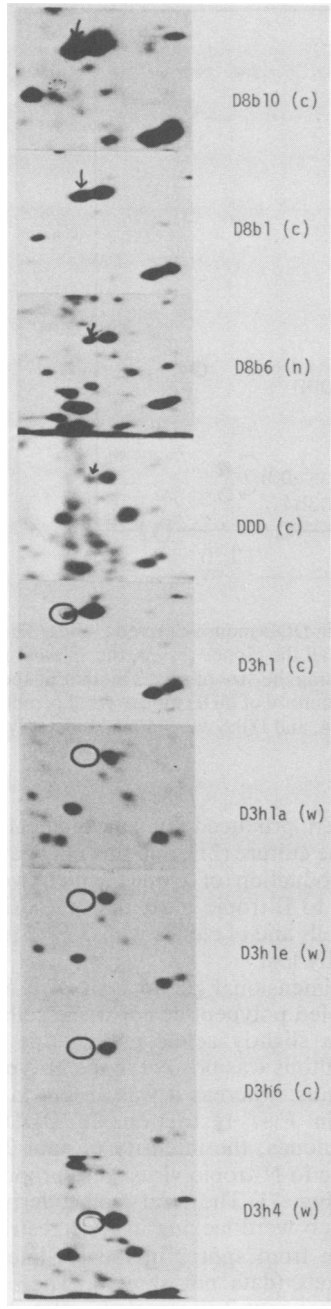

FIG. 6. Two-dimensional gel analysis of various DDD mouse-derived clones. Only the area in the vicinity of spot 2 is shown. The fractions tested were nuclear (n), cytosol (c), or whole (w) cell fractions as indicated in parentheses. In this fractionation, the nuclear fraction was not completely free from cytosol. D8b10, D8b1, D8b6, and DDD (primary culture of DDD mouse embryos) showed *Fv-1*^{n} restriction, whereas D3h4, D3h6, and D3h1 and its subclones D3h1a and D3h1e were free from *Fv-1* restriction. Spot 2 is indicated with an arrow when present and with a circle when the spot was faint or absent.

common difference was observed in two spots, spot 1 and spot 2 (Fig. 5). Spot 1 was present in the dually permissive cells, but not in the N-type cells of both origins. Spot 2 (molecular weight of around 20,000 daltons, pI of about 5.5) was present in N-type cells of both origins; in dually permissive G66m1 it was absent, and in D3h1 it was markedly reduced in comparison with the spot in D8b1. Though spot 1 was not well reproduced in subsequent studies, spot 2 was reproducibly identified on account of its position just beside the intense spot A which appeared in all preparations tested (Fig. 5). These polypeptide differences were reflected in both the nuclear (Fig. 5) and the cytosol fractions (D8b1 and D3h1 in Fig. 6).

To test the possible correlation between the intensity of spot 2 and *Fv-1* restriction, other DDD-derived clones were similarly analyzed (Fig. 6). All the clones devoid of *Fv-1* restriction were free from spot 2; the presence of the faint spot in the dually permissive D3h1 was probably due to insufficient cloning, as all the D3h1-derived subclones, all of which were dually permissive (D3h1a, D3h1e, D3h1j and D3h1m), were free from the spot. N-type clones and the uncloned DDD primary culture cells had spot 2. Interestingly, D8b1 and D8b10, which were more restrictive to B-tropic virus than the uncloned DDD primary culture cells, had spot 2 which was more intense, and a proportional relation between the intensity of spot 2 and the restriction to B-tropic virus was observed (Fig. 7).

To learn whether the absence of spot 2 in the dually permissive cells was due to the deletion of the polypeptide itself or to the mutation in the coding gene resulting in the replacement of methionine by other amino acids, we compared the autoradiograms with the silver-stained gels, which detect unlabeled proteins also. For this purpose, after the fluorography, 2,5-diphenyloxazole was removed by shaking the gels, first in water and then in dimethyl sulfoxide, until the gels became transparent; dimethyl sulfoxide was removed by shaking the gels in 30% methanol and 7% acetic acid. The gels were silver stained in the manner described (16). Figure 8 compares the autoradiograms with the silver-stained gels. Clearly, in the silver-stained gel of D3h1e, spot 2 was totally absent, indicating that polypeptide 2 was deleted in D3h1e cells. The autoradiogram and the stained gel of the congenic SIM.R strain (B type) (1) are also shown in Fig. 8. In the autoradiogram spot 2 was absent, whereas in the silver-stained gel an intense spot whose position approximately corresponds to that of spot 2 was observed. This may indicate that polypeptide 2 of SIM.R had little or no methionine or that its rate of synthesis was very low in this culture.

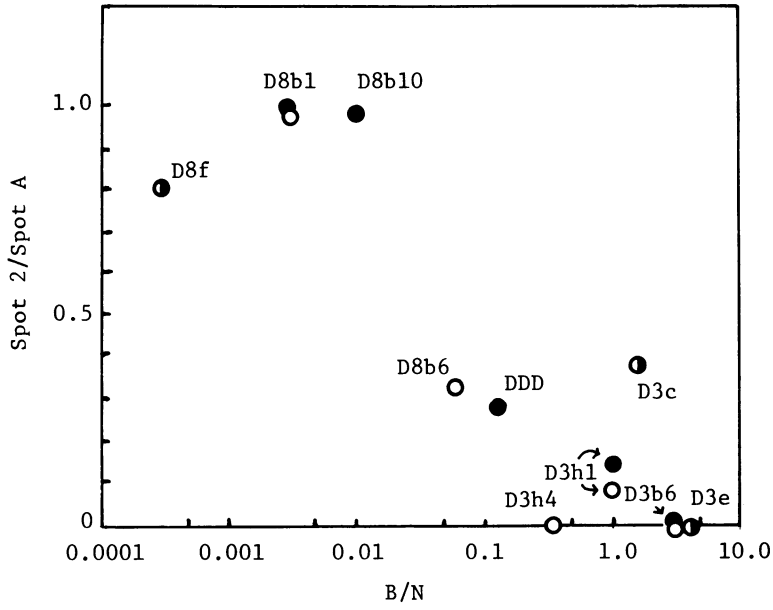

FIG. 7. Relation between *Fv-1*^{n} restriction and the size of spot 2 in DDD mouse-derived clones. The spot size was determined as described (11). As spot A appeared intensely in all the clones tested, the size of spot A was used as the standard. The spot 2/spot A ratio was calculated by dividing the size of spot 2 by that of spot A in the same gel. The B/N ratio was taken from Fig. 2. DDD was a primary culture of DDD mouse whole embryos; D3e, D3c, and D8f were cloned once, and D8b1, D8b10, D8b6, D3h1, D3h4, and D3h6 were cloned twice. Symbols: ○, cytosol; ●, nuclear fraction; ◑, whole cell lysate.

DISCUSSION

We isolated N-type and dually permissive cells from inbred DDD and G mouse strains (this report and reference 21) which differed in some genetic markers (15). In dually permissive cells of both origins, the *Gpd-1* locus closely linked to *Fv-1* was retained (this report and reference 21), and in G mouse-derived dually permissive clones an intact pair of chromosome 4 was present (21). The loss of *Fv-1* restriction was possibly brought about by changes not in *Fv-1* itself but rather in a gene(s) modifying the *Fv-1* phenotype (though we cannot completely exclude the opposite possibility). Genetic crossing indicated the presence of such a modifier gene(s) (this report and reference 2).

The mechanism of the segregation of the cells different in *Fv-1* restriction is interesting. The question is whether the uncloned DDD primary cultures were mere mixtures of the typical N-type and dually permissive cells or a uniform population of intermediate *Fv-1* restriction. We favor the latter alternative because, as reported previously, the phenotype of the mixture, as in the former alternative, was dually permissive (21). The segregation was probably brought about by somatic mutations. The intermediate *Fv-1*^{n} restriction in (DDD × C57L)F_1 (Fig. 4) may indicate a gene-dosage effect. Deviation in relative gene dosage, modifier versus *Fv-1*, may have been produced by karyological changes during the culture (21), and this may have resulted in production of clones which were more resistant to B-tropic virus than the original uncloned cells and of clones whose *Fv-1* restriction was totally lost.

Two-dimensional gel analysis of [^{35}S]methionine-labeled polypeptides of these cells indicated that a slightly acidic polypeptide of about 20,000 daltons was present in the N-type cells of both origins, whereas it was absent in the cells lacking in *Fv-1* restriction. In DDD mouse-derived clones, the intensity of spot 2 and the resistance to N-tropic virus were proportionally related (Fig. 7). The feral mouse-derived SC-1 cells which were lacking in *Fv-1* restriction (3) were free from spot 2 in the [^{35}S]methionine-labeled gels (data not shown). These findings suggest that spot 2 is somehow related to *Fv-1*^{n} restriction. However, in view of strong technical limitations in the two-dimensional gel analysis, i.e., detection of only a minor proportion of cellular polypeptides and inability to attribute any functions to the polypeptide in question, this statement is open to question. When the autoradiograms of various other mouse strains were compared, spot 2 was present both in the N-type C3H/He, NFS, and G and in the B-type BALB/c cells (data not shown), though it was undetectable in SIM.R (Fig. 7).

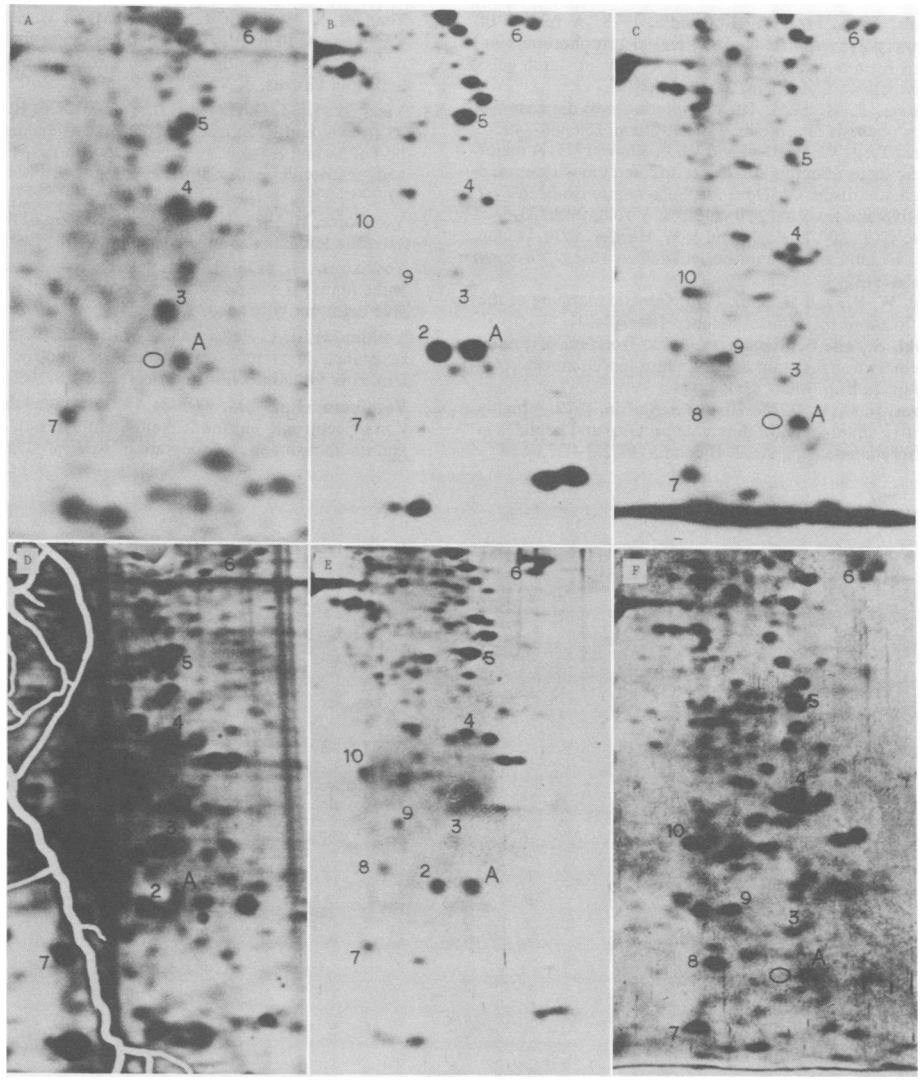

FIG. 8. Comparison of the autoradiograms with the stained gels: analysis of the whole cell lysates. (A) Autoradiogram of SIM.R. (B) Autoradiogram of D8b1. (C) Autoradiogram of D3h1e. (D) Silver-stained gel of SIM.R. (E) Silver-stained gel of D8b1. (F) Silver-stained gel of D3h1e.

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