VIRAL GENES INVOLVED IN LEUKEMOGENESIS

I. Generation of Recombinants between Oncogenic and Nononcogenic Mouse Type-C Viruses in Tissue Culture*

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Type-C RNA retroviruses are present in a wide variety of mammalian species. These viruses can be classified as endogenous or exogenous depending upon whether or not the complete virus genome is present within the germline of its species of origin. Although exogenous type-C viruses have often been shown to cause neoplastic diseases, much less is known about the oncogenic potential of endogenous viruses (1). There is also little understanding, as yet, of the mechanisms by which type-C viruses induce leukemia in the susceptible host.

We reasoned that the generation of recombinants between an oncogenic mouse type-C virus and an endogenous virus without demonstrable malignant potential could provide an important genetic approach for analysis of viral gene(s) directly involved in leukemogenesis. In this report, we describe the development of a tissue culture system that has made possible the reproducible generation of recombinant viruses. By genetic analysis of the recombinants obtained, regions of the viral genome involved in two distinct host restrictions to virus expression have been mapped.

Materials and Methods

Cell Culture. Cells were grown in Dulbecco's modification of Eagle's minimum essential medium supplemented with 10% calf serum (Colorado Serum Co., Denver, Colo.) in 60-× 15-mm plastic Petri dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). The cells used included clonal lines of continuous mouse cells BALB/3T3 and NIH/3T3 (2), and a normal rat kidney (NRK) line (3). Other mouse cell lines were derived from embryo cultures of a wild mouse (WM-C) from a colony maintained by M. Collins, Microbiological Associates, Bethesda, Md. and from the Fv-1 congenic Sim and SimR strains (4) provided by A. Axelrad, University of Toronto, Toronto, Canada. Embryo cultures of CBA, C3H, C57BL, and C57BL/10 (5) were also utilized. The human tumor, A673 (6), canine sarcoma, D17 (7), and the mink, MvILu (ATCC), cell lines have also been reported.

Viruses. A clonal isolate of Rauscher-murine leukemia virus (MuLV) and a temperature-sensitive (ts) mutant of this virus, ts 25, have been described (8). An inducible xenotropic endogenous virus, BALB: virus-2, was originally isolated after 5-iodo-2'-deoxyuridine induction of BALB/c embryo cells in tissue culture (9). The virus was cloned by two cycles of end point dilution in A673 cells before use in these studies.

Virus Assays. Infection of cells was performed according to previously published methods

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Abbreviations used in this paper: KiMuLV, Kirsten murine leukemia virus; MuLV, murine leukemia virus(es); NRK, normal rat kidney; PIU, polymerase-inducing units; rec, recombinant; ts, temperature sensitive; WM-C, mouse cell line derived from embryo cultures of a wild mouse.
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Type-C virus synthesis by infected cultures was measured by an assay for virion-associated reverse-transcriptase activity in tissue culture fluids (10). Neutralization tests were performed by the focus reduction method using MuLV pseudotypes of KiMSV (9).

Typing Radioimmunoassays for MuLV Proteins. The isolation of the p15, p12, p30, and gp70 structural proteins of Rauscher-MuLV and BALB:virus-2 as well as the reverse transcriptase and the gag gene-coded p10 proteins of Rauscher-MuLV have been described in detail (11–18). Each protein was labeled with 125I to high specific activity by the chloramine-T method (19) and used, along with caprine anti-Rauscher-MuLV and BALB:virus-2 sera, to develop appropriate type-specific competition radioimmunoassays.

Briefly, twofold serial dilutions of isopycnically purified recombinant viruses were incubated at 37°C for 1 h with limiting amounts of caprine hyperimmune antiserum in a 0.2-ml reaction mixture containing 10 mM Tris-hydrochloride (pH 7.8), 1 mM EDTA, 0.4% Triton X-100, 1% bovine serum albumin, and 20 mM (for p12 and p30 radioimmunoassays) or 250 mM (for p15, p10, reverse transcriptase, and gp70 radioimmunoassays) NaCl. 10^4 cpm of the corresponding 125I-labeled viral protein was added and incubated for 3 h at 37°C followed by an overnight incubation at 4°C. Saturating amounts of swine anti-goat IgG serum were added, incubated for 3 h at 4°C, and diluted with 0.8 ml of the above buffer in which bovine serum albumin was omitted. Immunoprecipitates were collected by centrifugation, supernates aspirated, and the radioactivity in the precipitate measured in a Searle 1285 gamma counter (Searle Radiographics Inc., Des Plaines, Ill.). Protein concentrations were determined as described by Lowry et al. (20).

Results

In Vitro Generation of Recombinants between Rauscher-MuLV and BALB:virus-2. We set out to obtain recombinants between a prototype ts mutant of the oncogenic Rauscher strain of MuLV (8, 21) and the endogenous xenotropic BALB:virus-2 (9). Use of these viruses permitted the design of a protocol for recombinant virus isolation based upon specific virus growth requirements (Fig. 1). By assay at 39°C on NIH/3T3 cells, the replication of the ts mutant and xenotropic parental viruses, respectively, was effectively blocked. However, recombinants possessing Rauscher-MuLV envelope functions and BALB:virus-2 sequences in genes affected by the ts lesions might be expected to grow efficiently in mouse cells at the nonpermissive temperature.

To generate potential recombinant viruses, we utilized a wild mouse embryo cell line, WM-C, which was permissive for replication of certain mouse xenotropic viruses. WM-C cells were first chronically infected with BALB:virus-2, and then superinfected with the Rauscher-MuLV ts mutant at the permissive temperature (31°C). The mutant utilized, ts 25, represented a class known to accumulate noncleaved gag gene precursors at the nonpermissive temperature, 39°C (22). To control for mutant leakiness or reversion, WM-C cells were infected with the mutant alone, and passaged under identical conditions. After 4 wk, virus released from each culture was tested for infectivity for NIH/3T3 cells at the restrictive temperature. Only if the infectivity of the virus at 39°C was significantly enhanced by passage of the mutant through WM-C cells replicating the xenotropic virus, were individual virus clones selected by the microtiter procedure for further analysis (Fig. 1).

Table I demonstrates the results of several experiments designed to generate recombinant viruses. It can be seen that ts 25 was stable, exhibiting no evidence of infectivity at 39°C after propagation for 4 wk at 31°C in WM-C cells. However, after its passage in BALB:virus-2-producing WM-C cells, virus infectivity for NIH/3T3 cells at the nonpermissive temperature ranged from 10^{5.5} to 10^{1.5} polymerase-inducing units (PIU)/ml compared to 10^{4.5} – 10^{5.0} PIU/ml at 31°C. Virus growth at 39°C could...
Fig. 1. Protocol for the in vitro isolation of type-C recombinant viruses between a ts mutant of Rauscher-MuLV and BALB: virus-2.

TABLE I

Frequency of Recombinants between Rauscher-MuLV ts 25 and BALB: virus-2

| Virus source | Virus titer at following temperature: | Percentage of Recombinants* |
|--------------|-------------------------------------|-----------------------------|
|              | 31°C  | 39°C  | Ratio 39°C/31°C | PIU/ml | %    |
| WM-C cells   |       |       |                |        |      |
| + BALB: virus-2 | <10⁶  | <10⁶  | --             | --     | --   |
| + wt Rauscher-MuLV | 10⁵.0 | 10⁸.0 | 10⁸.0         | 10⁵.0  | 10¹.0| 0.03 |
| + ts 25      | <10⁶  | <10⁶  | <10⁻⁵.⁰       | --     | --   |
| WM-C cells producing BALB:virus-2 + ts 25 |       |       |                |        |      |
| Exp. 1       | 10⁴.5 | 10¹.⁰ | 10⁻³.⁵         | 10⁴.5  | 10⁻³.⁵| 0.03 |
| Exp. 2       | 10⁵.⁰ | 10¹.⁵ | 10⁻⁵.⁰         | 10⁵.⁰  | 10⁻⁵.⁰| 0.03 |
| Exp. 3       | 10⁴.⁵ | 10¹.⁵ | 10⁻³.⁰         | 10⁴.⁵  | 10⁻³.⁰| 0.1  |
| Exp. 4       | 10⁴.⁵ | 10¹.⁵ | 10⁻⁴.⁰         | 10⁴.⁵  | 10⁻⁴.⁰| 0.01 |

Exponentially growing NIH/3T3 cells were infected at 31°C and 39°C with serial 0.5-log₁₀ dilutions of tissue culture fluids obtained from each virus-infected culture according to the protocol depicted in Fig. 1. The virus titer was defined by PIU/ml and was determined by the endpoint dilution for detection of virion-associated polymerase activity in 50-fold concentrates of tissue culture fluids obtained 3 wk after virus infection. Polymerase activity was measured by poly(rA)-oligo(dT)-directed poly(dT) synthesis according to methods previously described (10).

* Maximum frequency of recombinants was determined from the ratio of infectivity at 39°C/31°C.

have resulted from reversion of the mutant to wild type or to complementation or recombination with the xenotropic virus. Thus, assuming that all of the viruses capable of propagation at the nonpermissive temperature were true recombinants, the frequency of recombinants, as determined from the ratio of infectivity at the two temperatures, would be ~0.1% (Table I).

Immunological Identification of Recombinant Viruses. Type-specific antigenic determi-
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Fig. 2. Immunologic typing of the translational products of rec 25a-3 virus, a recombinant between Rauscher-MuLV ts25 and BALB: virus-2. Isopycnically purified rec 25a-3 virus was tested at twofold serial dilutions as a competing antigen in type-specific radioimmunoassays for Rauscher-MuLV, BALB:virus-2, retrovirus, and/or BALB:virus-2 proteins including: (A) p15; (B) p12; (C) p30; (D) p10; (E) reverse transcriptase; and (F) gp70. In control experiments, rec 25a-3 virus was tested in group-specific radioimmunoassays, in which anti-BALB:virus-2 serum was used to precipitate the corresponding 125I-labeled Rauscher-MuLV proteins. Protein concentrations were determined according to Lowry et al. (20).

Fig. 2 illustrates the results of analysis of one such virus, rec 25a-3, in type-specific radioimmunoassays for Rauscher-MuLV and BALB:virus-2 gag, pol, and env gene-coded proteins. In typing assays for the p15, p12, and p30 structural proteins of Rauscher-MuLV (Fig. 2A-C), the virus exhibited only limited competition. These results demonstrated the lack of immunologic identity of the p15, p12, and p30 proteins of rec 25a-3 with those of Rauscher-MuLV. In contrast, rec 25a-3 completely displaced the corresponding radioactive probe in each of the homologous radioimmunoassays for BALB: virus-2 p15, p12, and p30 gag gene-coded proteins (Fig. 2A-C). We have previously demonstrated that only the inducible xenotropic class of mouse endogenous viruses, of which BALB:virus-2 is the prototype, fully competes in

nants have been readily demonstrated in the p15 and p12 gag gene-coded proteins, as well as in the reverse transcriptase and envelope glycoprotein (gp70) of mouse leukemia viruses (13-15, 17, 18, 23). Furthermore, using appropriate antisera, type-specific determinants have also been demonstrated even in the more broadly immunoreactive proteins such as p30 (24) and p10 (M. Barbacid and S. A. Aaronson. Unpublished observations.). These antigenic determinants have been useful in identifying the origin of translational products of in vivo generated recombinants of mouse type-C viruses (25, 26).

To determine whether the viruses that grew in NIH/3T3 cells at 39°C were recombinants, clonal viruses were isolated from each potential recombinant stock, grown up to mass culture, purified by banding in sucrose gradients, and submitted to immunologic typing of their translational products. To achieve the greatest range of recombinants possible, only a few virus clones were analyzed from any given experiment.

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Fig. 3. Partial genetic mapping of in vitro generated recombinants between Rauscher-MuLV ts25 and BALB: virus-2 induced from their immunologic typing performed as described in Fig. 2 for rec 25a-3. Proteins exhibiting parental Rauscher-MuLV ([ ]) or BALB: virus-2 (■) antigenic determinants are indicated. In viruses depicting intracistronic crossing-overs, the relative localization and extent of Rauscher-MuLV and BALB: virus-2 derived genetic information has been arbitrarily assigned.

Thus, the results shown in Fig. 2 establish the presence of the BALB: virus-2 gag gene sequences in the rec 25a-3 viral genome.

We utilized a similar approach to analyze the envelope glycoprotein of rec 25a-3 virus. As shown in Fig. 2F, Rauscher-MuLV but not BALB: virus-2 type-specific determinants were detected in rec 25a-3 gp70. These results establish rec 25a-3 as a recombinant containing env gene sequences of Rauscher-MuLV and at least the 5' moiety of the gag gene of BALB: virus-2.

The translational products of rec 25a-3, including the gag gene-coded p10 protein and the reverse transcriptase, were also antigenically typed. Homologous immunoassays capable of detecting type-specific determinants of these proteins were only available for the corresponding Rauscher-MuLV products (16, 17). As shown in Figure 2D and E, neither p10 nor reverse transcriptase of rec 25a-3 virus were antigenically homologous with those of Rauscher-MuLV. Instead, they exhibited a competition pattern similar to that of BALB: virus-2. These results, along with those described above, argue that the sequences coding for the gag and pol gene of rec 25a-3 virus were derived from the BALB: virus-2 parent, whereas its env gene product, gp70, originated from Rauscher-MuLV.

We next submitted to immunological analysis 25 clonal viruses isolated according to the protocol shown in Fig. 1. 11 viruses were demonstrated to be recombinants between Rauscher-MuLV ts 25 and BALB: virus-2 (Fig. 3). The remainder were
Rauscher-MuLV revertants (5) or phenotypic mixtures of the parental viruses (9). The origin of the viral sequences of each recombinant virus was determined by the ability of its respective gene products to compete in the corresponding Rauscher-MuLV or BALB:virus-2 homologous radioimmunoassays as described for rec 25a-3 virus.

In some cases, a recombinational event was mapped within sequences coding for virion proteins. Fig. 4 illustrates the competition pattern of prototype recombinant viruses in the homologous Rauscher-MuLV reverse-transcriptase assay. Rec 25d-10 competed to a full extent, indicating that its polymerase originated from Rauscher-MuLV. In contrast, the competition pattern of another recombinant, rec 25c-3, was indistinguishable from that of BALB:virus-2, suggesting the contribution of its translatable pol gene sequences to that recombinant. Some recombinants, however, exhibited an intermediate competition pattern. As shown in Fig. 4, rec 25e-8 reverse transcriptase, although distinguishable from that of Rauscher-MuLV, more closely resembled it than the BALB:virus-2 enzyme (Fig. 4). These findings were tentatively interpreted as evidence for intracistronic recombination within the pol gene of the parental viruses (Fig. 3).

The lesion that prevents Rauscher-MuLV ts 25 from replicating at the nonpermissive temperature has been shown to involve processing of its gag gene-coded precursor polypeptide (22). From the partial genetic maps of the recombinants generated by this virus and BALB:virus-2 (Fig. 3), it was observed that only one protein, p10, was consistently provided by BALB:virus-2. These results, along with the known location
### Table II

**Host Range Properties of Recombinant Viruses Containing Rauscher-MuLV and BALB:virus-2 Genetic Sequences**

| Viruses            | Mouse | Human A673 | Canine D17 | Mink Mv1Lu |
|--------------------|-------|------------|------------|------------|
|                    | NIH/3T3 | BALB/3T3  |            |            |
| **Parental viruses** |       |            |            |            |
| Rauscher-MuLV      | $10^{4.6}$ | $10^{4.0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ |
| BALB:virus-2       | $<10^{0}$ | $<10^{0}$ | $10^{2.0}$ | $10^{2.0}$ | $10^{2.0}$ | $10^{2.0}$ |
| **Recombinant**    |       |            |            |            |
| rec 25a-3          | $10^{3.6}$ | $<10^{2.6}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ |
| rec 25a-8          | $10^{3.6}$ | $<10^{2.6}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ |
| rec 25b-14         | $10^{3.6}$ | $10^{3.0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ |
| rec 25c-3          | $10^{3.6}$ | $10^{3.0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ |
| rec 25c-6          | $10^{5.0}$ | $10^{5.0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ |
| rec 25c-14         | $10^{5.0}$ | $<10^{5.0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ |
| rec 25d-4          | $10^{5.0}$ | $<10^{5.0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ |
| rec 25d-10         | $10^{5.0}$ | $<10^{5.0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ |
| rec 25d-22         | $10^{5.0}$ | $<10^{5.0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ |
| rec 25d-25         | $10^{5.0}$ | $<10^{5.0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ |
| rec 25e-8          | $10^{5.0}$ | $<10^{5.0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ | $<10^{0}$ |

*Exponentially growing cultures were infected at serial 0.5-log₁₀ dilutions with each virus. The virus titer in PIU/ml was determined as described in Table I.*

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**Table III**

*Genetic Mapping of the Fv-1-determined Host Range Restriction in Recombinant Viruses*

| Virus               | Virus titer on the following assay cell* | Tropism | Origin of Recombinant p30† | Percent competition in the anti-AKR-K36/AKR MuLV p30 assay§ |
|---------------------|-----------------------------------------|---------|---------------------------|--------------------------------------------------------------|
|                     | Sim          | SimR        |                           |                                                              |
| Rauscher-MuLV       | $10^{3.0}$   | $10^{3.5}$  | NB                        | 100                                                          |
| KiMuLV              | $10^{4.5}$   | $10^{1.8}$  | N                         | 100                                                          |
| 1684                | $<10^{2}$    | $10^{2.5}$  | B                         | $<20$                                                        |
| BALB:virus-2        | $<10^{2}$    | $<10^{2}$   | Xeno                      | $<20$                                                        |
| Recombinants        |             |             |                           |                                                              |
| rec 25b-14          | $10^{2.5}$   | $10^{2.8}$  | NB                        | Rauscher-MuLV                                               |
| rec 25d-10          | $10^{3.5}$   | $10^{3.8}$  | NB                        | Rauscher-MuLV                                               |
| rec 25d-22          | $10^{3.0}$   | $10^{1.8}$  | NB                        | Rauscher-MuLV                                               |
| rec 25d-25          | $10^{3.5}$   | $10^{2.8}$  | NB                        | Rauscher-MuLV                                               |
| rec 25e-8           | $10^{4.0}$   | $10^{4.0}$  | NB                        | Rauscher-MuLV                                               |
| rec 25a-3           | $10^{2.5}$   | $10^{1.8}$  | N                         | BALB:virus-2                                                |
| rec 25a-8           | $10^{2.5}$   | $<10^{2.8}$ | N                         | BALB:virus-2                                                |
| rec 25c-3           | $10^{3.5}$   | $10^{1.8}$  | N                         | BALB:virus-2                                                |
| rec 25c-6           | $10^{4.0}$   | $10^{1.8}$  | N                         | BALB:virus-2                                                |
| rec 25c-14          | $10^{3.5}$   | $<10^{2.8}$ | N                         | BALB:virus-2                                                |
| rec 25d-4           | $10^{4.5}$   | $10^{1.8}$  | N                         | BALB:virus-2                                                |

* Exponentially growing cultures were infected at serial $0.5-10$ log dilutions with each virus. Virus titer in PIU/ml was determined as described in the legend to Table I.
† The origin of viral sequences coding for p30 was determined as described in the legend to Fig. 3.
§ Relative displacement of the $^{125}$I-labeled viral protein achieved by 1 µg of isopycnically purified virus when tested as competing antigens in the anti AKR-K36 mouse serum: $^{125}$I-labeled AKR-MuLV p30 radioimmunoassay (24).

Embryo cells ranged from $10^4$ to $10^5$ PIU/ml. Under the same conditions, these viruses titered $<10^2$ PIU/ml in B-permissive C57BL/6 or C57BL/10 cell lines. This pattern of infectivity was indistinguishable from that observed with Kirsten MuLV (Ki-MuLV), a prototype N-tropic virus.

As a final test that their NB- or N-tropic host ranges were under Fv-1 control, each recombinant virus was tested for growth in embryo cells of Sim and SimR strains, congenic at Fv-1 (4). As shown in Table III, the NB-tropic recombinants demonstrated very similar titers for infectivity in Sim and SimR cells. In contrast, each putative N-tropic recombinant virus demonstrated a preference for growth in Sim embryo cells, which possessed the N-permissive allele at Fv-1. These findings established that N- and NB-tropic phenotypes of the recombinants were under genetic control in Fv-1.

An effort was next made to correlate the presence of specific proteins of either parental virus with host range of the recombinant virus. As shown in Table III, each NB-tropic recombinant possessed Rauscher-MuLV p30 as determined by immunologic typing (Fig. 3). In contrast, all of the N-tropic recombinants contained BALB:virus-2 p30. There was no apparent correlation between recombinant virus host range and any other gag, pol, or env gene product analyzed (Fig. 3). Thus, it was possible to assign the site of Fv-1 action at or closely linked to the viral p30.

Recently, Tress et al. (30) have found that antiserum prepared in C57BL/6 mice against AKR K36 leukemia cells (31) possesses antibodies that specifically recognize
Table IV
Neutralization of Recombinant Viruses by Normal Mouse Sera

| KiMSV pseudotype | Tropism | Neutralization titer* of the following sera |
|------------------|---------|---------------------------------------------|
|                  |         | C57BL/6 | BALB/c |
| Parental         |         |         |       |
| Rauscher-MuLV    | NB      | <20     | <20   |
| BALB:virus-2     | Xeno    | 2,000   | 2,000 |
| Recombinants     |         |         |       |
| rec 25a-3        | N       | <20     | <20   |
| rec 25a-8        | N       | <20     | <20   |
| rec 25c-14       | N       | <20     | <20   |
| rec 25d-10       | NB      | <20     | <20   |
| rec 25e-8        | NB      | <20     | <20   |
| D17-3B3‡         | Xeno    | 1,000   | 2,000 |

* Neutralization tests were performed by the focus reduction method (32). Around 100 focus-forming units (ffu) of each helper virus pseudotype of KiMSV were incubated with serial dilutions of normal mouse serum for 30 min at 37°C before assay on NIH/3T3 or NRK cells. The number of foci was scored at 7-10 d. Results are presented as the reciprocal of the highest serum dilution giving 67% or greater reduction in the number of MSV foci.

‡ D17-3B3 is a previously described recombinant between Rauscher-MuLV and BALB:virus-2 (25).

the p30 proteins of MuLV exhibiting either N- or NB-tropic host range. We considered it of interest to determine whether the p30 antigenic determinants recognized by anti AKR K36 serum and apparently associated with N-tropism could be detected in the N-tropic recombinants, whose p30 protein was of xenotropic origin. Recombinant viruses were tested as competing antigens in the anti-AKR K36 serum: 125I-labeled AKR-MuLV p30 immunoassay (24). As shown in Table III, none of the recombinants possessing BALB:virus-2 p30, in spite of their N-tropic host range, exhibited significant competition. In contrast, the NB-tropic recombinants, which possessed Rauscher-MuLV p30, scored as positives in the same assay (Table III). These results indicate that the ability of each recombinant to compete in the anti-AKR K36 serum: 125I-labeled AKR-MuLV p30 was determined by the origin of their p30 protein rather than by their Fv-1 controlled host range.

Target of Mouse Serum Neutralizing Factor for Xenotropic Virus Maps in the env Gene. It has previously been shown that sera of many mouse strains contain high-titered neutralizing activity against xenotropic but not ecotropic type-C viruses (32). This factor has been demonstrated not to have an antibody nature, but instead to be associated with the low-density lipoprotein fraction of normal mouse serum (33). The availability of recombinants involving Rauscher-MuLV and BALB:virus-2 made it possible to map within the viral genome the target of another potent host restriction to endogenous virus expression.

We compared the ability of normal C57BL/6 and BALB/c mouse serum to neutralize the infectivity of KiMSV pseudotypes of recombinant viruses containing different representations of the genetic sequences of their respective parental viruses. As can be seen from the results in Table IV, both sera neutralized the BALB:virus-2 parental virus at high titer but had no effect on the infectivity of Rauscher-MuLV.
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The infectivity of each of the recombinant viruses involving ts 25 and BALB:virus-2 was also unimpaired. These viruses varied in their representation of genetic information of the parental viruses, but all encoded the env gene product of Rauscher-MuLV. A previously characterized recombinant, D17-3B3, was isolated from a stock of Rauscher-MuLV passaged in BALB/c mice (25). By typing immunoassays, this virus was shown to code for Rauscher-MuLV gag and pol gene products. However, its gp70 appeared to be a recombinant molecule containing antigenic determinants of both Rauscher-MuLV and BALB:virus-2 origin. As shown in Table IV, D17-3B3 was neutralized by normal mouse sera at the same high titers as BALB:virus-2. These results help to identify the env gene as the region within the xenotropic viral genome coding for the target of the neutralizing factor.

Discussion

This report describes an approach to mammalian type-C virus genetic analysis involving the generation of recombinant viruses in tissue culture. We have taken advantage of the temperature restriction of a Rauscher-MuLV mutant and the xenotropic host range of an endogenous mouse type-C virus to devise a double selection system for isolation of recombinant viruses. This has allowed the reproducible isolation of relatively large numbers of recombinants from parental viruses with very different biological properties. Furthermore, the fact that one of the parentals was a conditional mutant, potentially allows recombinants to be designed in almost any viral gene for which there exists a ts lesion.

In this study, we utilized Rauscher-MuLV ts 25, a mutant blocked in gag gene precursor polypeptide cleavage (22). All of the recombinants obtained were shown to replace, at least, the p10 protein of this virus with that of BALB:virus-2, thus mapping the lesion in ts 25 at the carboxy terminus of the viral gag gene (27). The inhibition of gag gene precursor polypeptide processing by a ts lesion located at its carboxy terminus could be interpreted as evidence for the existence of polarity in its maturation process. Alternatively, the ts lesion could cause an alteration of the tertiary structure of this polyprotein in such a way that normal processing is impaired.

The genomes of the recombinant viruses isolated in this study have been characterized by immunologic typing of the antigenic determinants present in their translational products (25). This allows the identification of most type-C viral proteins as belonging to a specific parent. Thus, we were able to unambiguously identify viruses as recombinants and construct a partial genetic map of their respective genomes. By this approach, it was possible to demonstrate frequent crossing-overs in many individual recombinants. Oligonucleotide fingerprinting analysis of avian type-C recombinant viruses have indicated that viable recombinants require the presence of sequences of the same parental virus at both 5' and 3' termini (34). If this were the case, the number of crossing-overs would range from two to as many as four among the different mouse type-C virus recombinants isolated in this study. The frequency of recombination was also determined. Frequencies ranged from 0.01 to 0.05%, at least 100-fold lower than those reported with avian type-C viruses (35, 36). These differences may be a result of the fact that the genomes of the parental avian viruses are much more closely related than those of the mouse type-C viruses utilized in our studies.

The Fv-1 locus is an important genetic restriction that affects endogenous virus expression and neoplasia (29). Intensive efforts to study the mechanism of Fv-1
restriction have led to understanding that it involves an intracellular block to virus replication and is exerted at a level before virus integration (37, 38). Many Fv-1 B-permissive mouse strains contain N-tropic endogenous viruses. The late appearance of B-tropic virus is often associated with the development of lymphoma (39). There is strong evidence that the B-tropic virus arises as a result of genetic alteration or recombination involving the N-tropic virus rather than from a distinct endogenous virus (26, 40, 41). Genetic as well as biochemical and immunological evidence have linked the Fv-1 gene restriction with the region of the viral genome coding for the major structural protein p30 (30, 42-45).

In our studies, analysis of the host range of a large group of mouse cell-tropic recombinants between the NB-tropic Rauscher-MuLV and xenotropic parental viruses revealed the acquisition of a host range phenotype distinct from that of either parent. Many recombinants exhibited N-tropic host range as defined not only by growth in NIH/Swiss and BALB/c cells but in other mouse strains, including Sim and SimR, congenic at Fv-1 (4). Our results demonstrated that preservation of NB-tropism by the recombinant was correlated with conservation of Rauscher-MuLV p30 and with no other known virus coded gene product. Thus, these findings further establish a target of Fv-1 restriction at or very near viral sequences coding for p30. Previous studies have demonstrated a very close immunologic relationship between the respective gag gene products of N-tropic and inducible xenotropic endogenous type-C viruses. These findings have suggested that the latter arose by recombination from the ecotropic virus relatively recently in evolution (25). The fact that the BALB: virus-2 gag gene p30 confers N-tropic host range to recombinant viruses capable of infecting mouse cells is evidence consistent with this concept.

A potent inhibitor of xenotropic virus expression in many mouse strains is a neutralizing factor (32) that resides within the low-density lipoprotein fraction of normal mouse serum (33). The mechanism of action of this factor is not yet understood. It was possible to demonstrate lack of neutralization by mouse serum of any of our newly generated recombinants, each of which shared only presence of sequences coding for Rauscher-MuLV gp70. Conversely, a previously isolated in vivo recombinant, in which a large portion of the Rauscher-MuLV env gene has been substituted by BALB:virus-2 sequences (25) was as effectively neutralized by mouse serum as the parental xenotropic virus. Thus, the site of action of this host control on xenotropic virus expression was demonstrated to map at the viral env gene.

Among the most important biologic questions pertaining to replication-competent type-C viruses concerns the mechanism by which they cause neoplasia. Recombinants generated and characterized in this report should be useful in determining what regions of the viral genome are essential for oncogenicity. In addition, such recombinants made it possible to determine whether endogenous xenotropic viruses possess malignant potential. In either case, in vivo testing of the oncogenic properties of a wide range of recombinants constructed as described in these studies should provide a useful strategy that may eventually allow localization of the putative "leuk" gene of the leukemia virus.

**Summary**

An approach toward elucidation of the mechanisms of action of mammalian leukemia viruses has been made by the generation in tissue culture of recombinant
viruses between a potent murine leukemia virus (MuLV), Rauscher-MuLV, and an endogenous xenotropic mouse type-C virus, BALB:viruse-2, without known malignant potential. Using a double selection system devised to select against the temperature-sensitive (ts) lesion associated with a mutant of Rauscher-MuLV and the xenotropic host range of BALB:viruse-2, recombinant viruses were obtained at frequencies ranging from 0.01 to 0.1%. Recombinant viruses were identified on the basis of the type specific antigenic determinants in the translational products of gag (p15, p12, p30, and p10 proteins), pol (reverse transcriptase), and env (gp70 glycoprotein) genes. By this approach, the partial genetic maps of a large number of recombinants were obtained. The fact that p10 of Rauscher-MuLV ts 25, the mutant utilized, was the only protein uniformly lacking in recombinant viruses, localized the lesion inhibiting gag precursor cleavage in this mutant at the carboxy terminus of its gag gene.

The recombinant viruses demonstrated two host range phenotypes as defined by Fv-1 host cell restriction. In each case, NB-tropic recombinants possessed the p30 of the Rauscher-MuLV parent, whereas the rest, N-tropic in host range, exhibited BALB:viruse-2 p30. Thus, it was possible to assign the site of Fv-1 action at, or closely linked, to the viral p30. The target within the viral genome of a second host restriction was also mapped. A serum factor, previously shown to specifically inactivate xenotropic virus infectivity, was demonstrated to exert its action on the viral env gene product. The system described here allows the generation of specific recombinant genotypes that should be useful in defining those regions of the viral genome involved in leukemogenesis.

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References

1. Aaronson, S. A., and J. R. Stephenson. 1976. Endogenous type-C RNA viruses of mammalian cells. Biochim. Biophys. Acta. 458:323.
2. Jainchill, J. L., S. A. Aaronson, G. J. Todaro. 1969. Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells. J. Virol. 4:549.
3. Duc-Nguyen, H., E. N. Rosenblum, and R. F. Zeigel. 1966. Persistent infection of a rat kidney cell line withauscher murine leukemia virus. J. Bacteriol. 92:1133.
4. Ware, L. M., and A. A. Axelrad. 1972. Inherited resistance to N- and B-tropic murine leukemia viruses in vitro: evidence that congenic mouse strains SIM and SIM-R differ at the Fv-1 locus. Virology. 50:339.
5. Stephenson, J. R., R. K. Reynolds, S. R. Tronick, and S. A. Aaronson. 1975. Distribution of three classes of endogenous type-C RNA viruses among inbred strains of mice. Virology. 54:53.
6. Giard, D. J., S. A. Aaronson, G. J. Todaro, P. Arnstein, J. H. Kersey, H. Dosik, and W. P. Parks. 1973. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J. Natl. Cancer Inst. 51:1417.
7. Riggs, J. L., R. M. McAllister, and E. H. Linnette. 1974. Immunofluorescent studies of RD114 virus replication in cell culture. J. Gen. Virol. 25:21.
8. Stephenson, J. R., and S. A. Aaronson. 1973. Characterization of temperature-sensitive mutants of murine leukemia virus. Virology. 54:53.
9. Aaronson, S. A., and J. R. Stephenson. 1973. Independent segregation of loci for activation of biologically distinguishable RNA C-type viruses in mouse cells. Proc. Natl. Acad. Sci. U. S. A. 70:2055.
10. Ross, J. E., E. M. Scolnick, G. J. Todaro, and S. A. Aaronson. 1971. Separation of murine cellular and murine leukemia virus DNA polymerases. *Nat. New Biol.* **231**:163.

11. Oroszlan, S., C. L. Fisher, T. B. Stanley, and R. V. Gilden. 1970. Proteins of the murine C-type RNA tumor viruses: isolation of a group-specific antigen by isoelective focusing. *J. Gen. Virol.* **8**:1.

12. Strand, M., and J. T. August. 1973. Structural proteins of oncogenic ribonucleic acid viruses. Interspec II, a new interspecies antigen. *J. Biol. Chem.* **248**:5627.

13. Strand, M., R. Wilsnack, and J. T. August. 1974. Structural proteins of mammalian oncogenic RNA viruses: immunological characterization of the p15 polypeptide of Rauscher murine virus. *J. Virol.* **14**:1575.

14. Tronick, S. R., J. R. Stephenson, and S. A. Aaronson. 1973. Immunological characterization of a low molecular weight polypeptide of murine leukemia virus. *Virology.* **54**:199.

15. Hino, S., J. R. Stephenson, and S. A. Aaronson. 1976. Radioimmunoassays for the 70,000 molecular weight glycoprotein of endogenous mouse type-C viruses: viral antigen expression in normal mouse tissues and sera. *J. Virol.* **18**:933.

16. Barbacid, M., J. R. Stephenson, and S. A. Aaronson. 1976. Structural polypeptides of mammalian type-C RNA viruses: isolation and immunologic characterization of a low molecular weight polypeptide. *J. Biol. Chem.* **251**:4859.

17. Krakower, J. M., M. Barbacid, and S. A. Aaronson. 1977. Radioimmunoassay for mammalian type-C viral reverse transcriptase. *J. Virol.* **22**:331.

18. Barbacid, M., and S. A. Aaronson. 1978. Membrane properties of the gag gene-coded p15 protein of mouse type-C RNA tumor viruses. *J. Biol. Chem.* **253**:1408.

19. Greenwood, F. C., W. M. Hunter, and J. S. Clover. 1963. The preparation of 131I-labeled human growth hormone of high specific radioactivity. *Biochem. J.* **89**:114.

20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **192**:265.

21. Stephenson, J. R., S. R. Tronick, and S. A. Aaronson. 1974. Temperature-sensitive mutants of murine leukemia virus. IV. Further physiological characterization evidence for genetic recombination. *J. Virol.* **14**:918.

22. Stephenson, J. R., S. R. Tronick, and S. A. Aaronson. 1975. Murine leukemia virus mutants with temperature-sensitive defects in precursor polypeptide cleavage. *Cell.* **6**:543.

23. Strand, M., and J. T. August. 1974. Structural proteins of mammalian oncogenic RNA viruses: multiple antigenic determinants on the major internal protein and major envelope glycoprotein. *J. Virol.* **13**:171.

24. Boiocchi, M., and R. C. Nowinski. 1978. Polymorphism in the major core protein (p30) of murine leukemia viruses as identified by mouse antisera. *Virology.* **84**:530.

25. Barbacid, M., K. C. Robbins, S. Hino, and S. A. Aaronson. 1978. Genetic recombination between mouse type-C RNA viruses: a mechanism for endogenous viral gene amplification in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* **75**:923.

26. Benade, L. E., J. N. Ihle, and A. Declave. 1978. Serological characterization of B-tropic viruses of C57BL mice: possible origin by recombination of endogenous N-tropic and xenotropic viruses. *Proc. Natl. Acad. Sci. U. S. A.* **75**:4553.

27. Barbacid, M., J. R. Stephenson, and S. A. Aaronson. 1976. gag gene of mammalian type-C RNA tumor viruses. *Nature (Lond.)* **262**:554.

28. Hartley, J. W., N. K. Woldorf, L. J. Old, and W. P. Rowe. 1977. A new class of murine leukemia virus associated with development of spontaneous lymphomas. *Proc. Natl. Acad. Sci. U. S. A.* **74**:789.

29. Lilly, F., and T. Pincus. 1973. Genetic control of murine viral leukemogenesis. *Adv Cancer Res.* **17**:231.

30. Tress, E., P. V. O'Donnell, N. Famulari, R. W. Ellis, and E. Fleissner. 1979. Polymorphism of B-tropic leukemia virus from BALB/c mice: association of a p30 antigen with N- is B-tropism. *J. Virol.* **32**:350.
31. Old, L. J., E. A. Boyse, and E. Stockert. 1965. The G (Gross) leukemia antigen. Cancer Res. 25:1813.

32. Aaronson, S. A., and J. R. Stephenson. 1974. Widespread natural occurrence of high titers of neutralizing antibodies to a specific class of endogenous mouse type-C virus. Proc. Natl. Acad. Sci. U. S. A. 71:1097.

33. Levy, J. A., J. N. Ihle, H. Olseko, R. D. Barnes. 1975. Virus-specific neutralization by a soluble nonimmunoglobulin factor found naturally in normal mouse sera. Proc. Natl. Acad. Sci. U. S. A. 72:5071.

34. Joho, R. H., M. M. Billeter, and C. Weissman. 1978. Concordance of the RNA termini of recombinants from crosses between avian retroviruses with different termini. Virology. 85:364.

35. Hanafusa, G. 1977. Cell transformation by RNA tumor viruses. In Comprehensive Virology. Volume 10. H. Fraenkel-Conrad and R. R. Wagner, editors. Plenum Press, New York. 401.

36. Vogt, P. K., and S. S. F. Hu. 1977. The genetics of RNA tumor viruses. Annu. Rev. Genet. 11:203.

37. Jolicoeur, P., and D. Baltimore. 1976. Effect of Fv-1 gene product on proviral DNA formation and integration in cells infected with murine leukemia viruses. Proc. Natl. Acad. Sci. U. S. A. 73:2236.

38. Sveda, M. M., and R. Soeiro. 1976. Host restriction of Friend leukemia virus: synthesis and integration of the provirus. Proc. Natl. Acad. Sci. U. S. A. 73:2356.

39. Peters, R. L., G. J. Spahn, L. S. Rabstein, G. J. Kelloff, and R. J. Huebner. 1973. Murine C-type RNA viruses from spontaneous neoplasms: in vitro host range and oncogenic potential. Science (Wash. D. C.). 181:665.

40. Robbins, K. C., C. D. Cabradilla, J. R. Stephenson, and S. A. Aaronson. 1977. Segregation of genetic information for a B-tropic leukemia virus with the structural locus for BALB: virus-1. Proc. Natl. Acad. Sci. U. S. A. 74:2953.

41. Rommelaere, J., H. Donis-Keller, and N. Hopkins. 1979. RNA sequencing provides evidence for allelism of determinants of the N-, B-, or NB-tropism of murine leukemia viruses. Cell. 16:43.

42. Pfeffer, L., T. Pincus, and E. Fleissner. 1976. Polymorphism of endogenous murine leukemia revealed by isoelectic focusing in polyacrylamide gels. Virology. 74:273.

43. Hopkins, N., J. Schindler, and R. Hynes. 1977. Six NB-tropic viruses derived from a B-tropic virus of BALB/c have altered p30. J. Virol. 21:909.

44. Faller, D. V., and N. Hopkins. 1978. T1 oligonucleotide maps of N-, B-, and NB-tropic murine leukemia viruses derived from BALB/c. J. Virol. 26:143.

45. Gautsch, J. W., J. H. Elder, J. Schindler, F. C. Jensen, and R. A. Lerner. 1978. Structural markers on core protein p30 of murine leukemia virus: functional correlation with Fv-1 tropism. Proc. Natl. Acad. Sci. U. S. A. 75:170.