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Murine Leukaemia Virus p30 Heterogeneity as Revealed by Two-dimensional Gel Electrophoresis Is Not an Artefact of the Technique

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SUMMARY

We have utilized two-dimensional (2D) gel electrophoresis [the first dimension being a linear pH gradient (5 to 8) and the second an 8 to 15% acrylamide gradient] to characterize the virion protein, p30, from several strains of purified murine leukaemia virus (MuLV). In all cases, we found that there was a predominant (70 to 90%) Coomassie Brilliant Blue-staining p30 spot, as well as several other species which differed in pI. The major p30 spot differed in pI among different MuLV strains and the minor spots varied depending on the host cell used to grow the virus. Specifically, (i) Moloney (M)-MuLV/NIH-3T3 showed two spots, a major one at pI 6.3 and a more acidic one, (ii) AKR/NIH-3T3, AKR/mouse embryo, and Gross/NIH-3T3 showed four spots, with the two basic, minor spots of AKR/NIH-3T3 appearing relatively decreased in intensity, and (iii) Rauscher (R)-MuLV/JLS-V9 (BALB/c) showed two spots, a major one with greater than 90% of the estimated Coomassie Brilliant Blue stain at a pI of 6.5 and a minor, acidic one. The major spots of AKR and M-MuLV viruses also differed in pI. The major spot of the AKR and Gross N-tropic viruses had a pI of 6.7 while that of NB-tropic virus M-MuLV had a pI of 6.3. The possibility that the heterogeneity observed in p30 was an artefact of the 2D gel technique had to be considered since urea was used to denature proteins in the first dimension of the gel. This possibility was made unlikely by our finding that another technique, chromatofocusing, gave the same results. Specifically, M-MuLV/JLS-V9 p30, when separated on chromatofocusing columns under non-denaturing conditions yielded three peaks, each of which directly corresponded to the three spots (pI: 6.1, 6.3, 6.6) observed on 2D gels. Furthermore, tryptic peptide maps of the major (pI 6.3) and one of the minor (pI 6.6) M-MuLV spots, although very similar in peptide composition, showed about five clearly defined differences. These results indicate (i) that the p30s of several N- and NB-tropic viruses are heterogeneous in pI, and (ii) for one particular MuLV, the p30 heterogeneity can be explained by a difference in amino acid composition. These findings of p30 charge heterogeneity may reflect either the presence of several different p30s in each virus particle and/or a heterogeneity in the virus population.

INTRODUCTION

In trying to understand how murine leukaemia virus (MuLV) particles are assembled, we and others have focused on the processing of Pr65\textsuperscript{gag}, the polyprotein precursor to the major core proteins. Specifically, cleavage of Pr65\textsuperscript{gag} gives rise to p30, the major capsid protein, as well as p15, a hydrophobic, membrane-associated protein and two nucleic acid-associated proteins, p12

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and p10 (Van Zaane et al., 1976; Arcement et al., 1976; Yoshinaka & Luftig, 1977; Eisenman & Vogt, 1978). Although the molecular details of this process are not yet understood, it appears that both a virus-associated protease (Yoshinaka & Luftig, 1977b) and a kinase that specifically phosphorylates p12 residues on Pr65$^{\text{gp70}}$ (Naso et al., 1979) are involved. The finding that Pr65$^{\text{gp70}}$ can be phosphorylated in vivo suggests that multiple charged forms should be found inside infected cells. Ledbetter (1979) indeed found such forms inside cells by using two-dimensional (2D) gels with non-equilibrium pH gradient gel electrophoresis (NEPHGE) in one dimension and SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in the other dimension. He also showed that multiple unique forms of p30 could be immunoprecipitated from both the cytoplasm of infected cells and virions. This result was somewhat unexpected and caused concern that the multiple p30 spots might be artefacts, since the proteins were denatured with urea prior to NEPHGE in the first dimension. For some proteins, this treatment can cause non-specific carbamoylation and modification of electrophoresed proteins (O'Farrell, 1975).

In order to assess the validity of the reported p30 heterogeneity, we have utilized an alternative non-denaturing procedure, chromatofocusing column chromatography, in conjunction with 2D gels to examine the p30s for a variety of N- and NB-tropic viruses. Our results suggest that the observed p30 heterogeneity is not an artefact of the preparative procedure.

**METHODS**

**Cells and viruses.** Moloney murine leukaemia virus (M-MuLV) was purified from MJD-54 cells, a line of JLS-V9 (BALB/c) mouse fibroblasts chronically infected with M-MuLV. The purification procedure utilized polyethylene glycol precipitation followed by sucrose density gradient centrifugation and has been previously described (Yoshinaka & Luftig, 1982). The MJD-54 cells were originally obtained from Dr K. Manly (Roswell Park Memorial Institute, Buffalo, N.Y., U.S.A.). The cell origin and designation of the remaining viruses used in this study are described in Table 1.

**Two-dimensional gel electrophoresis.** Purified virus or p30 protein was electrophoresed in two dimensions; the first was a linear non-equilibrium pH gradient (pH 5 to 8) where the gel consisted of 8.5% urea, 2% Nonidet P40 (NP40), 4% acrylamide–0.2% bisacrylamide and 2% of an Ampholine (LKB) mixture (1:4 of pH 3.5 to 10 and pH 5 to 8 Ampholines). The gel diameter was 0.45 cm and its length was 13 cm. Viral or protein samples (500 to 1000 μg protein) were dissolved in a lysis buffer of 8.5% urea, 2% NP40, 5% 2-mercaptoethanol and 2% of an Ampholine mixture in the same ratio as given above. The NEPHGE was for 1 h at 100 V, followed by 16 h at 400 V and 1 h at 800 V. After NEPHGE, the pH gradient of each gel was directly measured by using a miniature electrode (Gel Pro-pHiler; Bio-Rad) at 0.5 cm intervals along the gel length. Then the gel was immediately equilibrated with the second dimension sample buffer (0.0625 M-Tris–HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol) for 1 h and layered onto an 8 to 15% polyacrylamide gradient SDS slab gel with a 5% stacking gel. Further details of the 2D electrophoresis procedure are as described in O'Farrell (1975) and O'Farrell et al. (1977). Estimation of the relative percent of staining for the major p30 elliptical spots appearing on the 2D gels was done by measuring the area of the ellipses, using the formula $A = \pi ab$, where $a$ is the minor radius and $b$ the major radius. We assumed the staining intensity of the major and minor p30 spots was the same, although clearly the major spots have an increased intensity. Thus, the percent staining for the p30 major spots are relatively underestimated.

**Table 1. Properties of murine leukaemia viruses used**

<table>
<thead>
<tr>
<th>Virus designation</th>
<th>Cell line derivation</th>
<th>Tropism of virus</th>
</tr>
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<tbody>
<tr>
<td>(a) MuLV (AKR)</td>
<td>AKR (NIH-3T3)</td>
<td>N</td>
</tr>
<tr>
<td>(b) MuLV (AKR)</td>
<td>AKR embryo (NIH-3T3)†</td>
<td>N</td>
</tr>
<tr>
<td>(c) Gross MuLV</td>
<td>NIH-3T3</td>
<td>N</td>
</tr>
<tr>
<td>(d) Rauscher MuLV</td>
<td>JLS-V9 (BALB/c)</td>
<td>NB</td>
</tr>
<tr>
<td>(e) Moloney MuLV</td>
<td>NIH-3T3</td>
<td>NB</td>
</tr>
<tr>
<td>(f) Moloney MuLV</td>
<td>JLS-V9 (BALB/c) (MJD-54)</td>
<td>NB</td>
</tr>
</tbody>
</table>

* Viruses were obtained from the NCI. The virus was harvested from initially cloned, early passage chronically infected cell lines. Virus was purified from tissue culture fluids by double density-zonal gradient centrifugation and was received by us at -70 °C. The protein concentrations varied from about 0.8 to 2.1 mg/ml.

† AKR embryo refers to an embryo fibroblast cell line from AKR mice that was selected for spontaneous virus production and cloned.
Chromatofocusing column chromatography of M-MuLV p30. M-MuLV p30 was prepared from virions by molecular sieve column chromatography. M-MuLV (20 mg) purified from culture fluids of MJD-54 cells was disrupted with 2% NP40, 0.05 M-Tris–HCl pH 8.0, 10 mM-dithiothreitol (DTT) and the suspension was centrifuged for 20 min at 4 °C for 10000 rev/min in a Beckman J-21 centrifuge. The resulting supernatant (10 ml) was layered onto a Sephadex G-75 column according to Yoshinaka & Luftig (1977b). Five ml fractions were collected by eluting the Sephadex G-75 column with buffer (0.13 M-NaCl, 0.01 M-Tris–HCl pH 7.4, 10 mM-DTT) and the p30 peak positions as determined by SDS-PAGE were pooled and equilibrated to pH 7.4 with 0.25 M-imidazole–HCl, the chromatofocusing starting buffer. Then a chromatofocusing column was made by packing the Polybuffer exchanger PBE 94™ (Pharmacia) into a 1 cm diam. glass column after equilibrating the column with the pH 7.4 start buffer. The p30 sample from the Sephadex G-75 column was then loaded onto the column and was eluted with the elution buffer, pH 4.0 Polybuffer 74–HCl. Absorbance was monitored at 280 nm and the pH was measured for each eluted fraction.

Tryptic peptide analysis. Gel slices from appropriate fractionated and separated p30s on 2D gels were radio-iodinated, digested with trypsin and analysed on cellulose-coated thin-layer chromatography sheets as described by Elder et al. (1977).

RESULTS

Separation of MuLV p30s on 2D gels

Using the technique of NEPHGE followed by SDS–PAGE, we examined the heterogeneity of murine leukaemia virus p30s obtained from a variety of N- and NB-tropic viruses. We first examined the p30s of Moloney MuLV obtained from MJD-54 cells, a line of chronically infected JLS-V9 (BALB/c) cells. In the first dimension involving NEPHGE, the gradient went from pH 5 to 8 and, as seen in Fig. 1, three p30 spots were observed. The middle spot at pI 6.3 is clearly the major spot. Based on a semi-quantitative method, by which we measured the stained area of the spots, we estimated that the area of the major spot is 77% of the total p30 Coomassie Brilliant Blue stain. We noted that the same pattern seen in Fig. 1 for M-MuLV was obtained regardless
of whether the MJD-54 cells were in culture for a period of 1 year or were early passage (P24) cells. To study the frequency of p30 heterogeneity among different MuLV isolates, we next prepared 2D gels for five other viruses. The p30 regions were cut out for each and aligned according to pH values (Fig. 2). We noted that as with M-MuLV from MJD-54 cells, all virions showed a similar heterogeneous pattern of staining for p30, with one major spot and one or more minor spots. Based on the estimation method described in the text, we showed that the major spots had 82%, 73%, 72%, 91%, 74% and 77% of the total p30 staining in samples (a) to (f) respectively.

It can also be seen from Fig. 2 that the pl value for the major p30 spot of N-tropic viruses (a to c) was pl 6.7, while that of NB-tropic viruses (d to f) was pl 6.3 or 6.5. These values are consistent with the data of Schindler et al. (1981). In addition to the major p30 spot, all of the MuLVs showed one or more minor spots. We were concerned that such minor spots might be artefactually generated during the NEPHGE experimental procedure, i.e. isocyanate ions generated by decomposition of urea can result in carbamoylation of proteins. This led us to perform several additional experiments.
**MuLV p30 heterogeneity in 2D gels**

Control experiments using 2D gels

First, we incubated several known proteins in 8.5 M-urea at 37 °C for 10 to 14 h in order to see whether the specific heterogeneity observed in Fig. 2, where minor spots appear in addition to the major spot, could be generated. A random selection of frequently used mol. wt. marker proteins was utilized. The pattern seen in Fig. 2 could not be generated. Instead, no change, or a smearing of the spot (for phosphorylase B and ovalbumin) or a loss in intensity of the stained spot (for soybean trypsin inhibitor) was observed (our unpublished observations).

Then we asked whether the specific p30 spots from different MuLV preparations ran as predicted when different mixtures of viruses were run on 2D gels at the same time. The result shown in Fig. 3, where unlabelled M-MuLV (MJD-54) was mixed 1:1 with unlabelled Gross MuLV indicates that the mixtures ran as predicted. Thus, the arrows at the top point to the p30 spots of M-MuLV (MJD-54), and those at the bottom to the major p30 spots of Gross MuLV. All of the spots correspond exactly to those seen when the isolated viruses are run separately (see Fig. 2). Another mixing experiment using [35S]methionine-labelled M-MuLV (MJD-54) and unlabelled Gross MuLV showed a similar result, i.e. the pattern of major and minor p30 spots observed on an autoradiogram, when aligned with those on the corresponding Coomassie Brilliant Blue-stained gel using dots at the corners of the 2D gels, were shown to correspond to what was expected for M-MuLV (MJD-54) (autoradiogram) and Gross MuLV (Coomassie Brilliant Blue gel) (our unpublished observations).

Chromatofocusing experiments

Although we had indicated above that the p30 heterogeneity observed in Fig. 2 for 2D gels was unlikely to be caused by an artefact, another independent technique was needed, because proteins on the 2D gels were initially denatured with urea. M-MuLV (MJD-54) particles were therefore disrupted with 2% NP40 and subjected to Sephadex G-75 column chromatography (Yoshinaka & Luftig, 1977b) in order to obtain sufficient amounts of p30 for subsequent chromatofocusing experiments. The p30 peak obtained from the column was clearly separated from p15, gp70 and other lower mol. wt. virion proteins. It was then pooled, equilibrated with pH 7-4 start buffer and loaded onto a chromatofocusing column. After elution with pH 4-0 Polybuffer 74–HCl, 3 ml fractions were collected and both the A280 and pH were monitored for each fraction. It can be seen from Fig. 4 that the p30 of M-MuLV (MJD-54) separated into three peaks, which will be designated f-1, f-2 and f-3, with pls of 5-6, 5-2 and 5-0, respectively. The major peak (f-2) was at pl 5-2. These results correspond to the 2D system for M-MuLV (MJD-54) p30 (Fig. 1 and 2). A difference, however, is that the apparent pl value in the chromatofocusing experiments is 1-1 pH units lower than that in the 2D system. This is probably due to the Donnan potential effect which occurs as a result of Polybuffer exchange in the beads (Sluyterman & Wijdenes, 1978).

We next wanted to determine if the three chromatofocusing peaks (f-1, f-2, f-3) each corresponded on a 1:1 basis to the three 2D gel p30 spots. Thus, we isolated the p30 peaks (f-1, f-2, f-3) from the chromatofocusing column, removed the Polybuffer, lyophilized the protein and then ran each fraction on the 2D gel system. As seen in Fig. 5(a), the major fraction (f-2) indeed
Fig. 4. Fractionation of M-MuLV p30 by chromatofocusing column chromatography. The p30 fractions from a Sephadex G-75 column were pooled, equilibrated with the chromatofocusing start buffer and eluted from a Polybuffer exchange column with Polybuffer. The A_{280} and pH were measured for each of the 3 ml fractions. The three p30 peaks are f-1, f-2 and f-3, from left to right after the void volume.

Fig. 5. Two of the chromatofocusing column M-MuLV p30 peaks (f-1, f-2) seen in Fig. 4 were concentrated against Ficoll 400, Polybuffer was removed from p30 by a gel filtration step and the p30 fractions were then lyophilized before being separated by 2D (NEPHGE-SDS): (a) f-2, (b) f-1, (c) a 1:1 mixture of f-1 and f-2. Equal amounts of protein were used for each gel.

corresponds to the major spot (pI 6.3) seen in Fig. 2, while the minor fraction (f-1) corresponds to the minor spot at pI 6.6 (Fig. 5b). Similarly, f-3 was shown to correspond to the minor spot at pI 6.1 (data not shown). Furthermore, we found that when f-1 and f-2 were mixed together, the two p30 spots again migrated to their expected positions (Fig. 5c). Thus, we can say that there is a
Fig. 6. Autoradiograms of tryptic peptide maps of $^{125}$I-labelled gel spots obtained from the M-MuLV p30 f-1 and f-2 peaks seen in Fig. 5 and analysed according to Elder et al. (1977).
direct correlation between the chromatofocusing fraction peaks and the 2D gel p30 spots. We also note for Fig. 5(b, c) that the same amount of protein was loaded on to the 2D system for both the minor (f-1) as well as the major p30 species (f-2), giving the appearance that larger amounts of the minor species were present than there actually were in intact virus preparations (Fig. 2).

In another experiment, M-MuLV (NIH-3T3) p30 was chromatofocused as in Fig. 4 and 5, and both a minor p30 peak (pI 6.1) as well as a major p30 peak (pI 6.3) was observed (our unpublished data). Again, these peaks corresponded to what was observed in Fig. 2 for M-MuLV (NIH-3T3). This result further supports the proposed correlation between chromatofocused column peaks and 2D gel p30 spots.

Tryptic peptide maps

Since we had now demonstrated by two different techniques that there is a real difference in pI for the M-MuLV p30 fractions, we wanted to determine the basis for this difference. The f-1 and f-2 spots seen in Fig. 5 were cut out of the slab gel, labelled in vitro with $^{125}$I and tryptic peptide maps of these proteins were prepared. As seen in Fig. 6, although there is a great deal of similarity in the maps, there are five distinct differences between peptides observed for f-1 and f-2 (Fig. 6, circled spots). Thus, at least one reason for the different p30 pIs for the NB-tropic virus M-MuLV (MJD-54) may be attributed to differences in amino acid sequence.

DISCUSSION

Although it had been previously reported (Ledbetter, 1979), that the p30s of MuLVs exhibited charge heterogeneity when assayed by 2D-(NEPHGE/SDS-PAGE), there remained a concern that this heterogeneity might be attributable to an artefact inherent in the use of urea to denature proteins for NEPHGE. We have found this not to be the case. First, we confirmed and extended the earlier observations of Ledbetter (1979) to several other MuLV isolates and showed that all viruses exhibited p30 heterogeneity. Second, we also demonstrated by the use of non-denaturing chromatofocusing column chromatography that the pIs of MuLV p30s indicated charge heterogeneity. Finally, we showed that the tryptic peptide maps of a major (pI 6.3) and minor (pI 6.6) spot for one of the M-MuLV p30s, although exhibiting a great deal of similarity, also displayed about five differences.

Our results on charge heterogeneity of p30 are not unique to the N- or NB-tropic viruses used in this study. In Fv-1 restriction, SDS-PAGE studies have shown the virion protein target involved in B $\rightarrow$ NB tropism to be p30 (Hopkins et al., 1977; Gautsch et al., 1978). In fact, Schindler et al. (1981) showed that specific p30 tryptic peptide changes were associated with this conversion of tropism between B- and NB-tropic viruses. How the p30 changes are related to Fv-1 restriction, however, is not yet understood. For example, although p30 is a major structural protein of the virus core, only a small number of functional target molecules of p30 per virion are implicated in NB tropism (Kashmiri et al., 1977). This could be explained if minor species of p30 existed. Such species could arise, for example, if gag–pol precursors were processed differently than gag precursors, as a result of differential mRNA splicing in the p30 region.

It is also of interest that our study showed a difference in the pIs of the major p30 spots between R-MuLV and M-MuLV. Although these two viruses have some similar properties, i.e. they are replication-competent, do not cause transformation of tissue culture cells, and both induce leukaemia in newborn mice after a prolonged period of latency, they do also have differences, i.e. their gp70 sequences differ (Oroszlan et al., 1980) and they induce tumours in different subsets of mouse lymphoid cells (Reddy et al., 1980). Thus, the finding that there are different pIs for these two MuLV p30s should be considered along with known differences in gp70 and possible variability in the 5' or 3' long terminal repeated sequences when one is trying to understand how they induce different tumours.

It should finally be pointed out that the observation of multiply charged species for major virion capsid proteins is not unique. Bolen et al. (1981) have shown that VP$_1$, the major virus capsid protein of polyoma virus, when analysed by isoelectric focusing and SDS–PAGE has six distinct species and Nusse et al. (1980) found two species for p27, the major core protein of mouse mammary tumour viruses. It is thus entirely possible that charge heterogeneity is a
general phenomenon among viral capsid proteins. Furthermore, this may provide a mechanism whereby one can distinguish between vertex and other capsid polypeptides of icosahedral viruses, when there is only one polypeptide chain available. Thus, for MuLV, minor p30 species might be localized at vertices of the icosahedral core, while the predominant p30 species would constitute the major protein of the virus core shell. However, additional experiments are needed before we can discriminate between this alternative and the possibility that MuLV p30 charge heterogeneity reflects the presence of several different viruses in the virus population.

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