"sarc" Sequence Transcription in Moloney Sarcoma Virus-Transformed Nonproducer Cell Lines

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³H-labeled complementary DNA (sarc), complementary to the murine sarcoma virus (MSV)-specific portion of the Moloney MSV (M-MSV) genome, was prepared. M-MSV-specific RNA was then quantitated in the cytoplasm of several M-MSV-transformed, non-virus-producing, clonal NIH 3T3 cell lines. These lines, designated 71 N clones 5, 6, and 3, have been characterized previously by the degree to which they exhibit transformation properties and transcribe Moloney murine leukemia virus-related RNA (S. Salzberg and M. Green, *J. Virol.* 13: 1001-1004, 1974; N. Tsuchida and M. Green, *J. Virol.* 14:587-591, 1974). By the criteria of cell morphology and agglutination by concanavalin A, cells of clone 5 are highly transformed, cells of clone 6 are almost normal in the sense that they resemble the parent NIH 3T3 cells, and cells of clone 3 are phenotypically intermediate. In the present study, the amounts of cytoplasmic MSV-specific RNA correlated well with the relative degrees of transformation of the cell lines, varying over 35-fold between the least transformed (clone 6) and most transformed (clone 5) lines. Superinfection of either clone 5 or clone 6 with Moloney murine leukemia virus resulted in a fivefold increase in the MSV-specific RNA in the cell cytoplasm. Evidence from ³H-labeled complementary DNA:cell DNA hybridization studies indicated that the quantity of M-MSV-specific RNA in the nonproducer lines was not directly related to DNA provirus copy number in the cell DNA. Although clones 5 and 6 differ greatly in transformation characteristics and in MSV-specific RNA content, they each apparently contain about two copies of MSV-specific DNA sequence per haploid genome. Thus, factors such as site of provirus integration may be of primary importance in determining virus-specific transcription and cell transformation.

Recent studies in several laboratories have greatly advanced our knowledge of the structure of the Moloney murine sarcoma virus (M-MSV) genome as well as our knowledge of the origin of the nucleic acid sequences it contains. Mixtures of Moloney murine leukemia virus (M-MuLV) and M-MSV contain small RNA subunits of molecular weight 1.5×10^6 to 2.0×10^6 in addition to RNA subunits of molecular weight 3.0×10^6 to 3.4×10^6 , the latter size being characteristic of murine sarcoma virus (MSV)-free M-MuLV virus stocks (18, 21). Approximately 70% of the small, M-MSV-specific RNA subunit consists of sequences derived from the M-MuLV helper virus (about 50% of the total sequence content of M-MuLV) and approximately 30% of the sequences of this species are "extra" sequences in the sense that they are not present in M-MuLV (5, 8, 12, 16). Heteroduplex mapping

of the M-MSV subunit RNA from one virus clone (16) has elucidated the locations of the insertion of M-MSV-specific (extra) sequences as well as the specific deletions of M-MuLV sequences. Several groups (9, 19, 25) have prepared tritium-labeled DNA which is complementary to the M-MSV-specific region of the sarcoma virus genome [³H-cDNA(sarc)] and have used this DNA as a nucleic acid hybridization probe to study the origin of M-MSV-specific sequences and to monitor their presence in cellular RNA and DNA under a variety of conditions. The M-MSV-specific sequences of the M-MSV genome were found to be present in the DNA of uninfected mouse cells as a single copy per haploid genome (9-11). The most likely interpretation of this observation is that the M-MSV-specific sequence is a normal cell sequence which was incorporated into the M-MSV genome by a rare recombination event between M-MuLV and cell RNAs or between proviral DNA and cell DNA.

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Despite our knowledge about the structure of the M-MSV genome, the mechanism by which it causes transformation of cultured fibroblasts and sarcomas in animals is still unknown. Is transcription/translation of the integrated proviral MSV required for transformation, or is integration into cell DNA itself sufficient—perhaps integration at one or more specific sites? One useful approach to study such questions is to examine transcription of M-MSV sequences and the nature of M-MSV integration sites in transformed, nonproducer cell lines. In the present study, we have prepared ^3H -cDNA(sarc) and have used it as a hybridization probe to (i) analyze transcription of the M-MSV-specific sequences and (ii) determine the relative copy number of such sequences in the DNA of several M-MSV-transformed nonproducer cell lines (cloned) derived from NIH 3T3 cells. These transformed cell lines have been extensively characterized regarding degree of morphological transformation and agglutinability by concanavalin A (23). Transcription of overall virus-specific RNA in these cells has also been studied, although not with a probe which exclusively represented M-MSV-specific sequences (28). The particular cell lines chosen for this study exhibit great differences in their apparent degree of transformation by several criteria. It was thus possible to correlate the number of copies of M-MSV-specific transcripts in the cytoplasm with extent of transformation. In two of the cell lines, MSV-specific sequence copy number in the DNA was examined to ascertain if there is necessarily a correlation between provirus copy number and quantity of RNA transcripts.

MATERIALS AND METHODS

Cells and viruses. M-MSV-transformed S^+L^- cells (clone 319) were provided by A. E. Frankel and P. J. Fischinger. M-MuLV, free of MSV, was grown in high-passage mouse (Swiss) embryo cells (3); both the virus and cell stocks were originally provided by A. Hackett. M-MSV-transformed NIH 3T3 nonproducer cell lines (designated 71 N clones [CL] 5, 3, and 6) were obtained from G. Todaro. S^+L^- (clone 319) cells and their S^+L^+ (superinfected with M-MuLV) counterparts were grown in McCoy 5A medium, high-passage mouse embryo cells were grown in Eagle minimum essential medium, and NIH 3T3 derivatives were grown in Dulbecco-modified minimum essential medium. Fetal calf serum (10%) was used in all media.

Preparation of ^3H -labeled cDNA complementary to the M-MSV-specific (sarc) sequences. Growth and purification of viruses (5) and viral RNAs (4) have been described. Labeled cDNA probes were prepared by slight modifications of the procedures of Frankel et al. (12). S^+L^- (clone 319) cells, superinfected with M-MuLV, were used as a source of M-MSV(MuLV). After superinfection with M-MuLV (our strain of M-MuLV grown in high-passage mouse

embryo cells), progeny virus from clone 319 cells contained M-MSV and M-MuLV in a ratio of approximately 3 to 1 as estimated by focus and XC plaque assays (15, 22). Purified virus from these cells was used to synthesize ^3H -cDNA by the endogenous reverse transcriptase reaction. The reaction mixture consisted of 25 mM NaCl, 50 mM Tris-hydrochloride (pH 8.2), 6 mM dithiothreitol, 9 mM magnesium acetate, 1.0 mM each dATP, dCTP, and dGTP, 4.0×10^{-3} mM [^3H]dTTP (50 Ci/mmol), 100 μg of actinomycin D per ml, 0.008% Triton X-100, and approximately 0.5 to 1.0 mg of viral protein per ml. Typically, a 5-ml reaction mixture, incubated for 24 h, yielded 2.0×10^7 cpm of ^3H -cDNA. The product was purified as previously described (5). The labeled cDNA was then fractionated to obtain sequences present only in M-MSV, designated M-MSV specific or ^3H -cDNA(sarc). The fractionation scheme was similar to that of Frankel et al. (12). Briefly, total ^3H -cDNA was subjected to two consecutive cycles of hybridization with 60S RNA from M-MuLV and hydroxylapatite chromatography to remove all molecules which contained sequences complementary to M-MuLV. The remaining ^3H -cDNA was then hybridized to 60S RNA from M-MSV(MuLV) to provide a positive selection for labeled molecules capable of hybridizing to M-MSV. Specific modifications of the procedure of Frankel et al. (12) were as follows. Hydroxylapatite chromatography was conducted at 60°C rather than at 50°C. Concentration of cDNA-containing fractions and removal of phosphate (after hydroxylapatite chromatography) were achieved by precipitation with hexadecyltrimethylammonium bromide (20, 26). RNA (60S) from M-MSV(MuLV) (produced by 319 S^+L^+ producer cells) was used for the last, positive selection step in ^3H -cDNA(sarc) fractionation instead of RNA from the M-MSV(feline leukemia virus) pseudotype virus used by Frankel et al. This modification did not result in significant contamination of our ^3H -cDNA(sarc) probe with nonspecific mouse cell sequences [see Results, ^3H -cDNA(sarc) characterization].

Purification of cytoplasmic RNA and nuclear DNA from cultured mouse cells. After extensive rinsing with ice-cold 140 mM NaCl–0.5 mM MgCl_2 –3 mM KCl–10 mM Na_2HPO_4 –2 mM KH_2PO_4 (PBS), subconfluent, rapidly growing cells were removed from monolayers by scraping with a rubber policeman. The detached cells were further washed by three successive centrifugations through 100 volumes of PBS. Nuclei and cytoplasm were fractionated by methods described previously (17). The cells were lysed in buffer containing 0.14 M NaCl, 0.001 M MgCl_2 , 0.01 M Tris-hydrochloride (pH 8.5), and 0.5% Nonidet P-40 (Shell Oil Corp.). The intact nuclei were separated from the cytoplasm by centrifugation for 3 min at 2,000 rpm in an International PR-2 centrifuge.

Total cytoplasmic RNA was isolated, after digestion with 500 μg of proteinase K (EM Laboratories) per ml in 0.5% sodium dodecyl sulfate, by several extractions with phenol–chloroform–isoamyl alcohol (1:1:0.01, vol/vol/vol) at room temperature. After alcohol precipitation of the nucleic acids from the final aqueous phase, the RNA was dissolved in 0.01 M Tris-hydrochloride (pH 7.4)–0.001 M EDTA, at a final concn-

tration of 20 to 30 mg/ml as determined by a micro-quantitative orcinol reaction (30) and by measurements of absorbance at 260 nm.

Purified nuclei were washed with 0.1 M NaCl–0.01 M Tris-hydrochloride (pH 7.4)–0.001 M EDTA and then incubated at 37°C for 30 min in the presence of 500 µg of proteinase K per ml and 0.5% sodium dodecyl sulfate. Nucleic acids were extracted as described for cytoplasmic RNA except that large-bore pipettes were required to remove the viscous aqueous phases and more extraction steps were required to remove the proteins. After alcohol-precipitating the nucleic acids and redissolving them in 10 ml of 0.01 M Tris-hydrochloride (pH 7.4)–0.001 M EDTA, the solution was sonically oscillated in a glass scintillation vial under predetermined conditions which reduce the DNA to a length of 400 nucleotide pairs. RNA was digested by incubation for 5 h at 37°C in 0.3 N NaOH followed by neutralization with HCl. Removal of ribonucleotides and salts was accomplished by column chromatography (2.5 by 60 cm) with Sephadex G50 eluted with 0.01 M triethylamine bicarbonate. DNA in the appropriate column fractions was recovered by lyophilization and dissolved in 0.01 M Tris-hydrochloride (pH 7.4)–0.001 M EDTA at a concentration of approximately 14 mg/ml. The DNA was again denatured by heating to 100°C for 5 min and quick cooling just before use in hybridization experiments.

Analytical hybridizations. Hybridization reactions were conducted at 68°C in 30- to 35-µl volumes sealed in siliconized melting-point capillaries. Each capillary contained approximately 1,000 cpm of ³H-cDNA(sarc) (0.05 to 0.1 ng), a variable amount of unlabeled viral or cellular RNA (or an indicated amount of cellular DNA), 0.7 or 0.6 M NaCl, 0.05 M Tris-hydrochloride (pH 7.5) or 0.01 M PIPES [pH 6.7; piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (for cDNA:cell DNA hybridization), 0.002 M EDTA, and 50 µg of yeast tRNA. The sealed capillaries were heated to 100°C for 3 min and then placed directly in a 68°C water bath for incubation. All *C_t* or *C₀t* values shown are corrected values, approximately equivalent to hybridizations conducted under standard conditions of 0.18 M Na⁺ (6, 7). The degree of ³H-cDNA:RNA hybridization was determined by digestion with *S*₁ nuclease (24) at 45°C for 1 h followed by addition of 50 µg of double-stranded carrier DNA per ml, precipitation with 10% trichloroacetic acid, collection of the precipitate on cellulose nitrate membrane filters, and counting in toluene-based liquid scintillation fluor. The degree of hybridization of ³H-cDNA:DNA was determined by a batch hydroxylapatite procedure (14). After determining the absorbance at 260 nm of the 0.12 and 0.4 M phosphate buffer hydroxylapatite washes, the nucleic acids present in these washes were precipitated with 10% trichloroacetic acid and collected on filters. The dried filters were placed in glass scintillation vials, incubated at 100°C for 15 min in 0.5 ml of 0.5 N HCl, cooled, and dissolved in 0.5 ml of ethyl acetate. Finally, 20 ml of Aquasol (New England Nuclear) was added, and the samples were counted in a scintillation counter. The procedure for dissolving and counting the filters was found to minimize quenching of radioactivity due to nucleic acid precipitate and

filter. The procedure was necessitated by the presence of 100 to 300 µg of DNA in the hydroxylapatite buffer washes.

RESULTS

Characterization of the ³H-cDNA(sarc) probe. Tritium-labeled M-MSV-specific cDNA was isolated and characterized. After extensive hybridization with 60S RNA from M-MuLV to remove leukemia virus sequences (about 85% of the ³H-cDNA), 60 to 70% of the remaining ³H-cDNA hybridized to RNA from M-MSV-(MuLV). The overall recovery of M-MSV-specific ³H-cDNA, however, was only 3 to 5% due to cumulative losses of material during the isolation procedures. The ³H-cDNA(sarc) was tested for its specificity by hybridization with excesses of several viral and cellular RNAs (Table 1). Hybridization was quantitative to 60S RNA isolated from M-MSV(MuLV) and with cytoplasmic RNA prepared from the nonproducer MSV-transformed cell lines, clone 319 S⁺L⁻, and 71 N CL 5. In contrast, only a low degree of hybridization (10% or less) above background occurred to RNA isolated from purified M-MuLV virions or from 3T3 FL (uninfected mouse embryo) cells. These observations demonstrate that the ³H-cDNA(sarc) is indeed specific for M-MSV virus and M-MSV-transformed cells.

Quantitation of M-MSV-specific RNA in the cytoplasm of several transformed non-producer cell lines. Several cloned isolates of M-MSV-transformed, nonproducer cells (derived from transformed NIH 3T3 cells) were

TABLE 1. *Base sequence specificity of the M-MSV ³H-cDNA(sarc) probe*

RNA source	% ³ H-cDNA hybridization ^a
M-MSV(MuLV) 60S virion RNA	100
M-MuLV 60S virion RNA	9
M-MSV-transformed clone 319 S ⁺ L ⁻ cells	100
71 N CL 5 transformed, nonproducer cells	97
Uninfected, mouse 3T3 FL cells	10
Yeast tRNA	0 ^b

^a Equivalent *C_t* values for hybridizations involving viral RNAs and cellular RNAs were 50 and 10,000 mol·s/liter, respectively.

^b The ³H-cDNA(sarc) probe was approximately 5% resistant to nuclease *S*₁ after long incubation with or without yeast tRNA. This resistance presumably represents self-annealing and has been subtracted from *S*₁-resistant counts of all samples before determining hybridization percentages. *S*₁ resistance of samples containing MSV RNA was 100% of input counts; therefore no corrections, other than that for self-annealing, were applied.

provided by G. Todaro. These cell lines, 71 N CL 5, 71 N CL 3, and 71 N CL 6, exhibit different degrees of transformation as judged by observations of morphology and agglutination by concanavalin A (23). Cells of the line 71 N CL 5 are highly transformed—they are rounded and do not adhere well to surfaces; they are agglutinated to a great extent by concanavalin A. Cells of the line 71 N CL 6 exhibit very little evidence of transformation. With respect to morphology and agglutinability, they closely resemble the parent line, NIH 3T3. Cells of the line 71 N CL 3 are intermediate in transformation characteristics, although they resemble cells of 71 N CL 5 more than cells of 71 N CL 6.

Figure 1 shows the kinetics of hybridization of ^3H -cDNA(sarc) with 60S RNA from purified M-MSV(MuLV) (ratio of MSV to MuLV was 3:1 by bioassays) and with cytoplasmic RNAs from the various cell lines. Hybridization of the labeled cDNA(sarc) probes to unlabeled viral or cellular RNA was performed under conditions of considerable RNA excess. The amount of ^3H -cDNA was 0.05 ng and the added cellular RNA ranged from 2 to 200 μg . Even assuming the lowest possible percentage of MSV-specific RNA in the cells compatible with the data, in no sample could the hybridization reaction have depleted the unlabeled, sarcoma-specific RNA

by more than 10% of its original level. Under these conditions, ^3H -cDNA:RNA hybridization is a pseudo-first-order reaction in which the rate of reaction is inversely proportional to the genetic complexity of the RNA. Kinetic data are represented on semilogarithmic graphs, plotting the fraction of ^3H -cDNA hybridized as a function of $\log C_r t$ (the product of initial RNA concentration and the time of incubation) (1). The fraction of an RNA mixture (i.e., cellular RNA) complementary to ^3H -cDNA(sarc) can be estimated by dividing the $C_r t_{1/2}$ value determined for it by the "intrinsic" $C_r t_{1/2}$ value obtained by using only RNA complementary to the ^3H -cDNA(sarc) probe [$C_r t_{1/2}$ is the value at which one-half of the ^3H -cDNA(sarc) is hybridized]. The observed $C_r t_{1/2}$ value for hybridization with 60S RNA from M-MSV(MuLV) was 0.15. By making several assumptions, we calculate the apparent intrinsic $C_r t_{1/2}$ value of 0.026 for hybridization of ^3H -cDNA(sarc) and the M-MSV-specific RNA sequences in the 60S RNA. The assumptions utilized in this calculation are as follows: (i) the ratio of MSV RNA subunits to MuLV subunits is 3:1 as indicated by the bioassay results; (ii) the respective molecular weights of the MSV and MuLV subunits are 1.8×10^6 and 3.3×10^6 , respectively (16, 21); and (iii) the molecular weight of the MSV-specific region of the sarcoma virus genome (subunit) is 0.5×10^6 (8, 16). Thus, the fraction of sarcoma-specific sequences in the 60S RNA would be 0.174, and the intrinsic $C_r t_{1/2}$ would be 0.15 $(0.174) = 0.026$.

Assuming that (i) the intrinsic $C_r t_{1/2}$ value for hybridization of ^3H -cDNA(sarc) with M-MSV-specific RNA is 0.026 and (ii) the amount of cytoplasmic RNA per mouse cell is 1.0×10^{-5} μg (13; our average value determined from our cytoplasmic RNA recoveries was 1.0×10^{-5} $\mu\text{g}/\text{cell}$), the data of Fig. 1 indicate that 71 N CL 5 cells contain 1,114 copies of M-MSV-specific RNA in the cytoplasm, 71 N CL 3 cells contain 492 copies, and 71 N CL 6 cells contain 31 copies. Thus, the relative amounts of M-MSV-specific RNA in the cytoplasm of each of the transformed nonproducer lines correlates well with the relative degree of transformation they exhibit. These relative amounts of MSV-specific RNA are valid regardless of whether or not the actual copy numbers are strictly correct. It can be seen from Fig. 1 that the hybridization of ^3H -cDNA(sarc) with RNA from 71 N CL 6 does not follow the same kinetic pattern as the other lines. The significance of this is unclear, but it appears that some portions of the MSV-specific sequence are present in greater abundance than other portions in the cell RNA.

The effect of superinfection with M-MuLV on

FIG. 1. Quantitation of M-MSV-specific RNA transcripts in the cytoplasm of transformed, nonproducer lines. Samples contained 0.05 ng of ^3H -cDNA(sarc) and a vast excess of viral (0.01 to 0.1 μg) or cellular (1 to 300 μg) RNA in 30 to 35 μl of 0.7 M NaCl-0.05 M Tris-hydrochloride (pH 7.5)-0.002 M EDTA. Samples containing M-MSV(MuLV) 60S RNA were incubated for various times to achieve the desired $C_r t$ values. The samples containing cellular RNA were incubated for 40 h. The extent of hybridization was measured as fractional resistance of the labeled cDNA to digestion by *Aspergillus oryzae* nuclease S_1 . A background of 6% resistance to S_1 nuclease was observed in controls and was subtracted from the data for each sample.

M-MSV-specific transcription was investigated in lines 71 N CL 5 and 71 N CL 6. Cells of these lines were infected with M-MuLV at a multiplicity of infection of 3. The cells were passed two times in monolayer bottles and finally in roller bottles to get enough cells for nucleic acid extraction. Substantial virus production was confirmed by measurement of reverse transcriptase-containing particles in the medium. The M-MuLV-infected 71 N CL 6 cells contained approximately 159 copies of MSV-specific RNA per cell in the cytoplasm, an increase of about fivefold over the copy number in uninfected 71 N CL 6 (Fig. 2). Cytoplasmic M-MSV-specific RNA was likewise increased approximately fivefold by infection of line 71 N CL 5 with M-MuLV. Table 2 summarizes the relative M-MSV-specific RNA copy numbers in the various cell lines.

Quantitation of sarcoma virus-related sequences in the cellular DNAs of normal and M-MSV-transformed nonproducer cell lines. The reiteration frequency of sarcoma virus-related cell DNA sequences was studied with lines 71 N CL 5, 71 N CL 6, and NIH 3T3. This was accomplished by hybridization kinetic experiments in which the single-stranded ^3H -cDNA(sarc) probe was hybridized with a 1.5×10^6 or 3.0×10^6 -fold excess of unlabeled cell DNA. The results are shown in Fig. 3. From

FIG. 2. Increase in MSV-specific RNA in transformed, nonproducer cells after superinfection with M-MuLV. Cell lines 71 N CL 6 and 71 N CL 5 were superinfected with M-MuLV at a multiplicity of infection of 3. After passing the cells two times, cytoplasmic RNA was isolated from each line (1 to 1.5 weeks after infection) and analyzed for M-MSV-specific sequences as described in the text and in the legend to Fig. 1. The dashed lines represent data from Fig. 1 replotted here for comparison with the data for the superinfected cells.

TABLE 2. Summary of M-MSV-specific RNA quantitation in the cytoplasm of transformed mouse cell lines

Source of RNA	Equivalent $C_{r1/2}$ (mol·s/liter)	"sarc"-specific RNA ^a (% of total RNA)	"sarc" sequence equivalents per cell ^b
60S M-MSV(MuLV) RNA	0.15	17.4	
71 N CL 5	280	0.009	1,114
71 N CL 3	630	0.004	492
71 N CL 6	10,000	0.0002	31
71 N CL 5 (infected with M-MuLV)	50	0.052	6,221
71 N CL 6 (infected with M-MuLV)	2,000	0.0013	159

^a $[C_{r1/2} \text{ intrinsic for MSV-specific sequences} (= 0.026)/C_{r1/2} \text{ for cellular RNA}] \cdot 100$.

^b Assuming 10^{-5} μg of RNA per cell and 8.3×10^{-13} μg of RNA per "sarc" sequence equivalent.

data for 71 N CL 5, it can be seen that the hybridization curve for ^3H -cDNA(sarc) is dependent on the ratio of cell DNA to ^3H -cDNA. At 5 mg of cell DNA per ml, the final degree of probe hybridization is about 30%, whereas with 10 mg/ml the final degree of hybridization was about 45%. Hybridization reactions of ^3H -cDNA(sarc) with 10 mg of cell DNA per ml from 71 N CL 5 and 71 N CL 6 yielded indistinguishable kinetic patterns (45% final extent; $C_{0t1/2}$ for probe, slightly less than 1,000 mol·s/liter). These experiments were conducted twice and were quite reproducible. In contrast to the results with the nonproducer lines, probe hybridization in the presence of 10 mg of DNA per ml from uninfected NIH 3T3 cells reached an extrapolated maximum hybridization value of 27%. In each experiment, reannealing kinetics for the cell DNA was determined, although only one representative curve is shown in Fig. 3. In all cases, the $C_{0t1/2}$ values for reannealing of unique sequence DNA were 1.5×10^3 to 2.0×10^3 mol·s/liter. These values were determined by fitting the data points with a second-order kinetics curve and estimating $C_{0t1/2}$ by inspection.

We cannot determine precisely what the sarcoma virus-related copy numbers are from our data because, in the absence of complete probe hybridization, comparisons of $C_{0t1/2}$ values are imprecise. We can be reasonably certain that none of the lines contain more than four "sarc" DNA copies. Furthermore, two other conclusions are strongly suggested by our data: (i) lines 71 N CL 5 and 71 N CL 6 contain equivalent amounts of sarcoma virus-related sequences (probably only two copies per haploid genome), and (ii) line NIH 3T3 contains fewer copies of these sequences (probably one copy per haploid genome). Thus, the 35-fold difference in the

FIG. 3. Hybridization of ^3H -cDNA(sarc) to cellular DNAs. Three hundred micrograms (or $150\ \mu\text{g}$) of cell DNA was incubated for various times with $0.1\ \text{ng}$ of ^3H -cDNA(sarc) in sealed capillaries. The aliquot volume was $30\ \mu\text{l}$. The degree of annealing was determined by hydroxylapatite chromatography. Symbols: (●) 71 N CL 5, $10\ \text{mg}$ of cell DNA per ml; (○) 71 N CL 6, $10\ \text{mg}$ of cell DNA per ml; (△) 71 N CL 5, $5\ \text{mg}$ of cell DNA per ml; (▲) NIH 3T3, $10\ \text{mg}$ of cell DNA per ml; (□) representative reassociation of total cell DNA.

amount of cytoplasmic M-MSV-specific RNA in lines 71 N CL 5 and 71 N CL 6 is not reflective of quantitative differences in sarcoma-related sequences in the DNA.

Two factors are undoubtedly responsible for the low final extent of hybridization of our ^3H -cDNA(sarc) probe: (i) the probe was 190 nucleotides long (data not shown), and thus it was one-half to one-third the length of the reassociating cell DNA; and (ii) the sarcoma virus-related sequences in the cell DNA were probably present in only a two- to fourfold molar excess over the labeled probe (calculated assuming one to two copies of "sarc" per haploid genome). Because of the "length effect," it is quite reasonable to expect that the second-order rate constant (k) for annealing of the labeled probe would be two to three times smaller than that for self-annealing of the cell DNA since, in the range of 100- to 400-nucleotide chain length, k may be directly proportional to length or in any case to the square root of the length (29). Both the reduced rate constant and the lack of significant excess of cell DNA have the effect of reducing the final extent of hybridization of the probe. The quantitative effect of these factors has been analyzed (27; A. Tereba, Ph.D. thesis, University of Washington, Seattle, 1973; A. Tereba, manuscript in preparation). Assuming a rate constant for probe hybridization threefold less than that for cell

DNA reannealing and a two- to fourfold molar excess (on a gene basis) of cell DNA, these theoretical analyses predict 30 to 50% final probe hybridization values and a strong dependence of the final hybridization extent on the ratio of cell DNA to ^3H -cDNA. Thus, our data are reasonably consistent with expected kinetic patterns.

DISCUSSION

Nucleotide sequences complementary to M-MSV-specific cDNA were quantitated in the cytoplasmic RNA and nuclear DNA of several MSV-transformed nonproducer clones derived from NIH 3T3. Specific clones tested exhibit a wide range of transformation properties (23, 28). The present study demonstrates that the degree of transformation correlates well with the amount of M-MSV-specific RNA in the cytoplasm of each of three clones tested, implying that the steady-state level of MSV sequences (and possible translation of a specific polypeptide) is directly related to transformation. Whether or not this apparent proportionality between degree of transformation and M-MSV-specific RNA copy number is a generality must be tested by studies of more such M-MSV-transformed cell lines.

In our study, the amount of MSV-specific RNA in the cytoplasm of two clones was not related in a simple manner to the number of MSV-specific copies in the cell DNA. Cells of the 71 N CL 5 line contained approximately 35 times more MSV-specific RNA than cells of line 71 N CL 6 despite the fact that the two lines contain about the same number of DNA copies for this sequence. Perhaps the location of M-MSV-specific DNA integration is the determining factor in the rate of the MSV-specific RNA transcription and degree of transformation. Alternatively, the turnover rate for MSV-specific RNA may be drastically different in the lines, thus accounting for different steady-state levels. These possibilities have not yet been tested. The nonproducer clones utilized in this study should prove to be excellent systems for studying the role of integration site location for the following reasons. Unlike cells of many S^+L^- or nonproducer clones (11, 19) 71 N CL 5 cells possess a low MSV-specific DNA copy number, and yet they are very highly transformed in the absence of replicating helper virus. Thus, it is unnecessary to amplify MSV sequence copy number (and thus complicate matters) by adding replicating helper virus (11) to achieve complete transformation. For purposes of comparison, 71 N CL 6 cells possess a similar MSV-specific DNA copy number but low MSV-specific RNA content and minimal transformation.

Superinfection of lines 71 N CL 6 and 71 N CL 5 with M-MuLV increased the amount of MSV-specific RNA in the cytoplasm fivefold. This effect has been reported previously by Peebles et al. (19). However, it is important to note that superinfection of 71 N CL 6 did not bring MSV-specific RNA amounts up to the level of the other clones, although it did somewhat alter its morphological appearance. Thus, in our experiments superinfection with MuLV did not overcome the main transformation restriction (probably transcriptional block) in this cell line. Salzberg and Green (23) did report that such superinfection increased the agglutinability of 71 N CL 6 cells by concanavalin A.

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