Polymerase-defective mutant of the Bryan high-titer strain of Rous sarcoma virus

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ABSTRACT

A mutant of the Bryan high-titer strain of Rous sarcoma virus defective in reverse transcriptase is known as type alpha (BH-RSV alpha). BH-RSV alpha virion particles do not contain any polymerase-related proteins but they direct the synthesis of a normal sized Pr180 gag-pol polyprotein precursor in infected cells. Using a bioassay for polymerase gene function that is based on the requirement of viral replication for transformation of transfected chicken cells, we have localized the defect to the 2.5 kb EcoRI-KpnI DNA fragment containing more than 90% of the polymerase gene by comparison with the corresponding DNA fragment from the wild-type polymerase-positive BH-RSV, called type beta. In vitro recombination experiments with the polymerase gene of Schmidt-Ruppin RSV allowed us to map the defect to the 0.86 kb XbaI-BglII DNA fragment of the BH-RSV alpha polymerase. DNA sequence analysis of the entire polymerase gene of BH-RSV alpha and beta has revealed one point mutation that maps within that XbaI-BglII fragment and substitutes leucine in BH-RSV alpha for glutamine in the wild-type BH-RSV beta.

INTRODUCTION

The Bryan high-titer strain of Rous sarcoma virus (BH-RSV) was isolated after successive passages of RSV in chickens to select a virus that could rapidly induce tumors (1,2). BH-RSV was later shown to be replication defective (3,4), and the deletion of its env sequence was documented (5,6). The precise extent of the env deletion was determined by analyzing a molecular clone of BH-RSV obtained from proviral DNA (7). BH-RSV-infected cells produce viral particles that are deficient in the envelope glycoprotein. These virions are noninfectious; however, when introduced into cells by fusion they establish infection and cause cell transformation (8,9). Since BH-RSV does not encode the envelope protein, this virus, called BH-RSV(-), was used extensively to elucidate the function of the env gene products and

was also useful to show the existence of endogenous viral glycoproteins in certain cells (8,10).

In earlier studies, stocks of BH-RSV were found to contain nonconditional polymerase-defective mutants (10). Approximately 5-10% of the viral particles in BH-RSV stocks were negative for the presence of reverse transcriptase (11,12), and thus were unable to produce infectious viruses even when introduced into chicken cells expressing the endogenous virus glycoprotein. These viral particles became infectious only when both reverse transcriptase and envelope glycoprotein were provided in trans by double infection with a replication-competent helper virus. These mutant viruses from BH-RSV were named alpha type (10), whereas the wild-type BH-RSV were called beta type viruses.

In this report, we present a characterization of the BH-RSV alpha mutation. After testing a series of in vitro constructed recombinants using a transfection assay, we localized the mutation to a small fragment within the pol gene of the virus. We have shown that one amino acid within this region is substituted in the mutant when compared with the wild-type BH-RSV beta virus. This mutation does not appear to affect the synthesis of Prl80 gag-pol polyprotein precursor but it blocks the incorporation of this protein into virions. In addition, we have found that the pol gene of BH-RSV beta is more active in supporting viral replication than that of Schmidt-Ruppin strain of RSV (SR-RSV).

MATERIALS AND METHODS

Protein Studies

For metabolic labeling with isotope, chicken embryo fibroblasts (CEF) grown in 60 mm plates were incubated for 4 hrs in 1 ml of methionine-free Minimal Essential Medium (MEM) (GIBCO) containing 100 μ Ci of 35 S-methionine (1200 Ci/mmol, Amersham). The cultures were lysed in 400 μ l of RIPA buffer (13).

To isolate labeled virion particles, medium was collected from cultures labeled for 24 hrs in 2 ml of methionine-free MEM containing 200 μ Ci of 35 S-methionine and then for 12 hrs in 1 ml of complete medium. Harvested medium was centrifuged (8,000 rpm, 20 min, Sorvall, S34 rotor) to remove cell debris, and the clarified supernatant was layered over 2 ml of 20% sucrose and cen-

trifugation at 45,000 rpm for 45 min (Beckman SW50.1 rotor), resulted in pelleting of labeled virion particles.

Rabbit antiserum raised against total virion proteins was a gift from Dr. R. Feldman, The Rockefeller University; antiserum against avian myeloblastosis virus reverse transcriptase was obtained from the Office of Program Resources and Logistics of the National Cancer Institute. Preabsorbed serum was prepared by adding 20 μ g of protein from purified Rous associated virus-2 (RAV-2) virions in RIPA buffer for each μ l of anti-pol serum, incubating at 4 °C for 60 min, and spinning out the precipitate for 15 min in an Eppendorf Centrifuge. Immunoprecipitations were performed essentially as described earlier (13,14). Polyacrylamide gels were according to Laemmli (15).

Molecular Cloning of BH-RSV Proviral DNAs

Cloning of the wild-type BH-RSV proviral DNA from virus-infected 3Y1 rat cells and its characterization by restriction enzyme analysis have been previously described (16). In this report we designate the wild-type virus as beta to distinguish it from the polymerase-defective alpha type mutant. The proviral DNA of the BH-RSV alpha was isolated and molecularly cloned by the same strategy as in the case of the BH-RSV beta (7,16). In both cases the proviral DNAs were isolated from nonproducer (NP) clones. Details of the procedures for molecular cloning and for plasmid DNA isolation were as previously described (7,17).

Enzyme Reactions and Gel Electrophoresis

Restriction endonucleases were obtained from New England BioLabs or Bethesda Research Laboratories and were used as recommended by the manufacturer. Nick translation, ligation, removal of 5' phosphate, and 5' and 3' end-labelings of DNA fragments were performed according to standard procedures (17). Gel electrophoresis of DNA and filter hybridizations were carried out as previously described (6,17).

Transfection of CEF with Viral DNA

Transfection was carried out according to the procedure of Wigler et al. (18). Preparation of CEF and transfection of SR-RSV cloned DNA were performed as previously described (19). Usually, cells were passaged on the third day after transfection, overlaid 24 hrs later with Ham's F-10 medium containing

0.4% of agar, and monitored for the appearance of characteristic foci.

DNA Sequencing

The chemical cleavage method of Maxam and Gilbert (20) was used throughout. Analysis of DNA sequences for the presence of restriction enzyme cleavage sites was facilitated by use of the computer program SEARCH (21).

RESULTS

Viral Proteins in BH-RSV Alpha-Infected Cells and Proteins in BH-RSV Alpha Virions

Nonproducer clones of CEF infected with BH-RSV alpha, free of helper virus, were isolated and the viral proteins were examined. Figure 1, panel A, shows the results of immunoprecipitation of viral proteins from extracts of BH-RSV alpha-infected cells using the anti-virion or anti-pol sera. Immunoprecipitates from RAV-2-infected cells run in parallel lanes are shown as controls. In all four NP clones examined (two of which are shown in Fig. 1), a band which comigrates with the RAV-2 Pr180 was precipitated by both anti-virion and anti-pol sera. These results indicate that BH-RSV alpha, although defective in polymerase activity, can direct the synthesis of a normal-sized gag-pol polyprotein precursor.

Figure 1 shows the results of immunoprecipitations of virion proteins. As reported earlier (22), RAV-2 virions did not have the Pr180 precursor, but instead contained two subunits of the polymerase, p95 and p63, which were detected by immunoprecipitation. In contrast, polymerase related proteins were not detected in the virions released from two NP clones of BH-RSV alpha (Fig. 1B). In the case of each of these clones there was sufficient production of viral particles, as evidenced by the precipitation of gag proteins upon reincubation of the supernatant with anti-virion serum (Fig. 1C), ruling out lack of virus production as the cause of the negative result. This confirms earlier results (12,22) in which a radioimmunoassay failed to detect any pol related products in BH-RSV alpha virions.

Construction of SR-RSV/BH-RSV Chimeras

We sought to determine whether the defect is actually in

Figure 1. A: BH-RSV alpha proteins in normal CEF and in infected cells. Cell lysates were prepared from cultures that had been metabolically labeled with ^{35}S -methionine. CEF, normal CEF; R-2, RAV-2 infected culture; 1 and 2, independent BH-RSV alpha NP clones. Immunoprecipitation was performed with either anti-virion (V) or preabsorbed anti-pol serum (P). B, C: BH-RSV alpha proteins in purified virions. B: Purified ^{35}S -methionine labeled virion particles were immunoprecipitated with preadsorbed anti-pol serum. C: The material that did not precipitate was reincubated and precipitated with anti-virion serum. Although the amounts of virus released from the analyzed clones were not normalized, the autoradiograms shown in panels B and C, when overexposed for 3 months, did not show any bands comigrating with the polymerase proteins (data not shown). Electrophoresis was performed on 5-15% gradient (A), 7.5% (B), or 10% (C) SDS-polyacrylamide gels. The positions of molecular weight markers run in parallel lanes of the gels are shown in kilodaltons on the left side for (A) and in the middle for (B) and (C). The bands corresponding to polymerase precursor and gag precursor are identified as Pr180 and Pr76, respectively. The bands corresponding to reverse transcriptase are identified as p95 and p63.

the pol gene since the BH-RSV alpha viral genome directs the synthesis of an apparently normal Pr180 precursor (7). The biological activity of the BH-RSV alpha pol gene was tested in a transfection assay. As a control, the activity of the BH-RSV beta pol gene was examined in a similar manner. The transfection assay of CEF as described earlier (19) was used. Briefly, two SR-RSV molecular subclones, pSR-REP and pSR-XD2 (19), each

containing only part of the RSV genome, were ligated prior to transfection to generate a structure resembling an intact SR-RSV genome. pSR-REP contains the SR-RSV 5' LTR, gag, and pol genes, and the gp85-portion of the env gene; pSR-XD contains the SR-RSV gp37-portion of env, the src gene, and the 3' LTR. Neither plasmid alone can replicate and neither one can cause transformation of CEF since viral replication is required for stable transformation of transfected cells (19). Therefore it is possible to use morphological transformation as a marker for assaying biological activity of pol. Construction of in vitro recombinants between the pSR-REP clone and pBH-alpha or pBH-beta clones resulted in the generation of viral DNAs that contain all the regulatory sequences from SR-RSV and a pol gene derived from either BH-RSV alpha or beta. The strategy for construction of these recombinants is shown in Fig. 2. The EcoRI-KpnI fragment contains the gag-pol junction and more than 90% of the pol gene. This restriction fragment was excised from pBH-alpha or pBH-beta DNA by complete digestion with KpnI and EcoRI. The excised fragment was ligated to pSR-REP DNA from which the pol-containing EcoRI-KpnI fragment had been removed by complete digestion with KpnI and by partial digestion with EcoRI. DNA of the in vitro-constructed pol recombinants was used to transform E.coli; colonies were screened for plasmids of the desired construction. Since both BH-RSV strains contain an XhoI site in pol which is not present in SR-RSV, and since BH-RSV beta contains an extra BamHI site in pol which is not present in BH-RSV alpha and SR-RSV (7), these two enzymes were used to determine the origin of the EcoRI-KpnI pol fragment in the recombinants. The resulting recombinants, pSR-pol-alpha and pSR-pol-beta, have the structures shown in Fig. 2.

Mapping the Defect to the pol Coding Sequences.

DNA from pSR-REP, or the recombinants pSR-pol-alpha or pSR-pol-beta, was ligated to pSR-XD2 DNA and transfected onto CEF as described (19). Replacement of the SR-RSV pol gene with the BH-RSV alpha pol gene abolished focus-forming ability, while replacement of the SR-RSV pol gene with that of BH-RSV beta did not affect focus-forming ability. Therefore, the defect that is responsible for the lack of the reverse transcriptase enzyme in

Figure 2. Construction of SR-RSV/BH-RSV chimeric clones. **A:** Structures of molecular clones that were used to derive the chimeric constructions. **B:** Products of *EcoRI* and *KpnI* restriction enzyme digestions. **C:** Structures of resulting recombinants. The positions of relevant restriction enzyme cleavage sites are shown. For simplicity, in (C), and also in Figs. 3 and 4, the *EcoRI* and *KpnI* sites are shown at the 5' and 3' (with respect to *pol* RNA) borders of *pol*. E, *EcoRI*; K, *KpnI*; Xh, *XhoI*; Ba, *BamHI*.

BH-RSV alpha virion particles maps within the *EcoRI*-*KpnI* fragment of BH-RSV alpha.

Localization of the Mutation to a Defined Region of *pol* Gene

The *XbaI* and *BglII* cleavage sites within the *EcoRI*-*KpnI*

Figure 3. Construction of recombinants within *pol*. A: Structure of molecular clones that were used to derive the recombinants. B: Products of *Sst*I-*Xba*I or *Sst*I-*Bgl*II double digestions described in the text. C: Structures of resulting recombinants. In (A) and (B) the positions of relevant restriction enzyme cleavage sites are shown. Ss, *Sst*I; Xb, *Xba*I; Bg, *Bgl*II. Other symbols as in Fig. 2.

fragment divide the 2.7 kb fragment into three fragments of 1.07, 0.86 and 0.76 kb. Figure 3 shows the strategy utilized to map the pol defect to one of these three fragments. pSR-pol-alpha and pSR-pol-beta were used as parentals. The DNAs were linearized by complete digestion with SstI and partial digest conditions were used to generate SstI-XbaI or SstI-BglII fragments from pol (Fig. 3). The SstI-XbaI and SstI-BglII larger ("vector") and smaller ("insert") fragments from pSR-pol-alpha

TRANSFECTION OF *POL* RECOMBINANTS

(-)	(+)			
EXPECTED RESULT IF LESION IS IN:			ACTUAL RESULT	
<i>P</i>	<i>O</i>	<i>L</i>	Foci / Plate
+	-	-	0
-	+	+	300
+	+	-	450
-	-	+	0

Figure 4. Transfection of *pol* recombinants. The structure and biological activity of the *pol* gene of pBH-alpha (upper left) and pBH-beta (upper right) are shown for reference. Before transfection, each of the four constructs (shown in Fig. 3) was ligated to the pSR-XD2 DNA as described earlier (19). The schematic structure of the *pol* genes of the four recombinants derived (Fig. 3) and the results in a transfection assay are shown in the table. The actual results shown here are the average of the results of transfection of 3 independent molecular clones having the structure shown, except the third row, which is the average of the results of two clones.

and pSR-pol-beta were purified separately. In order to remove undigested or nicked circular plasmid (i.e., parental) DNA that might comigrate with the vector fragments, purified vector fragments were digested with *HpaI*, which cleaves parental DNA at a single site and is absent in the two vectors. For both the *SstI*-*XbaI* and *SstI*-*BglII* digestion products, the pSR-pol-alpha vector was ligated to the pSR-pol-beta insert and the pSR-pol-beta vector was ligated to the pSR-pol-alpha insert, resulting in the construction of the four *pol* recombinants illustrated in Fig. 3. A *Bam*HI restriction site, which differentiates the BH-RSV alpha- and beta-derived inserts, was used to verify the constructions.

The four recombinants shown in Fig. 3 were used in the DNA

transfection assay to map the pol defect to one of the following: the 1.07 kb EcoRI-XbaI ("P") fragment, the 0.86 kb XbaI-BglII ("O") fragment, or the 0.76 kb BglII-KpnI ("L") fragment. As shown in Fig. 4 the four recombinants that were constructed would be expected to yield a unique array of results depending on whether the mutation mapped in "P", "O", or "L". (Other unique patterns of results which would be expected if more than one mutation existed are not shown in the figure).

When the four recombinants were tested in the transfection assay, successful transformation of CEF correlated with a beta-derived "O" region only. Conversely, inability to produce foci on CEF correlated with an alpha-derived "O" region irrespective of the origin of the "P" or "L" fragments (Fig. 3). Thus, the defect in BH-RSV alpha is due to a mutation or mutations in the coding sequence corresponding to an 859 bp XbaI-BglII DNA fragment in the central portion of the pol gene.

Differences Between the pol Gene of BH-RSV alpha and beta; Comparison with the pol Gene of PR-RSV

Direct DNA sequence analysis of the entire pol gene of BH-RSV alpha and beta (including the gag portion starting at the EcoRI site) has revealed, apart from a few silent mutations, only one nonconservative point mutation that maps in the 859 bp XbaI-BglII fragment indicated by the recombination experiments described above. Adenine in the BH-RSV beta pol gene at position 3455 (the numbering is that utilized for polymerase of the Prague strain of RSV (PR-RSV) according to Schwartz et al., (23)) is substituted with thymine at the equivalent position in BH-RSV alpha. As a result of this mutation, the polar glutamine residue in the beta strain is substituted by nonpolar leucine in alpha.

Comparison of the BH-RSV pol sequences with the pol sequence of the PR-RSV has revealed the following: (i) pol genes of PR-RSV and of BH-RSV alpha both have a thymine at position 3455; (ii) seven amino acid changes distinguish both BH-RSV alpha and beta pol sequences from the pol sequence of PR-RSV. A summary of all the differences in the BH-RSV alpha and beta pol genes in comparison with the pol gene of the PR-RSV is presented in Tables 1 and 2.

Table 1.
Amino Acid Substitutions in the Polymerases Encoded by
PR-RSV, BH-RSV alpha, and BH-RSV beta

Differences in DNA and amino acid sequences ^a			
Nucleotide number	PR-RSV	BH-RSV alpha	BH-RSV beta
2345 ^b	Leu(TTG)	Val(GTG)	Val(GTG)
2600,2601	Ala(GCG)	Gly(GGC)	Gly(GGC)
3022	Ser(TCT)	Ala(GCT)	Ala(GCT)
3151	Val(GTG)	Ile(ATC)	Ile(ATC)
3455	Leu(CTG)	Leu(CTG)	Gln(CAG)
3725	Lys(AAG)	Arg(AGG)	Arg(AGG)
4520	Val(GTA)	Ala(GCA)	Ala(GCA)
5126	Gly(GGA)	Glu(GAA)	Glu(GAA)
5131	Thr(ACC)	Ala(GCC)	Ala(GCC)

a/ Strategy of the DNA sequencing and copies of the autoradiograms are available on request. At positions corresponding to the sequence variations in PR-RSV (23), the following bases were found in BH-RSV alpha and beta pol sequences: at position 2323 is T, 2416 is T, 2549 is G, 2589 is T, 2646 is G, 2649 is C, 2752 is T, 2818 is C, 3028 is C, 3075 is T, 3097 is A, 3413 is A, 3923 is T, 3948 is A, 4020 is T, 4080 is G, 4105 is A, 4464 is C, 4584 is C and at 5107 is G.
b/ Numbering according to Schwartz et al., (23). The number corresponds to the substituted nucleotide shown in the parentheses. First entry in the table shows a mutation that maps in the gag portion.

Differences Between the pol Gene of SR-RSV and BH-RSV beta.

Both SR-RSV and BH-RSV beta have wild-type pol genes that could be expected to be equally active in a transfection assay. Surprisingly, it appeared that pSR-pol-beta, the construct in which the pol gene of SR-RSV is replaced with that of BH-RSV beta, is more active in the transfection assay than the parental pSR-REP. Before transfection each of the constructs was ligated to the pSR-XD2 as described earlier (19). pSR-pol-beta consistently (in 5 independent experiments) caused rapid and complete transformation of the cultures in 5 days, while under the same conditions cells transfected with pSR-REP did not begin to show discernable foci until day 6, and the plates did not become completely transformed until day 7.

This observation was confirmed by measurement of the kinetics of production of infectious viral particles by chicken cells transfected with either pSR-REP and pSR-XD2 or with pSR-pol-beta

Table 2
Conservative Changes in the Polymerases Encoded by
PR-RSV, BH-RSV alpha and BH-RSV beta

Differences in DNA sequences							
Nucleo- tide number	PR- RSV	BH- RSV alpha	BH- RSV beta	Nucleo- tide number	PR- RSV	BH- RSV alpha	BH- RSV beta
2338 ^a	T	C	C	3504	T	C	C
2341	T	G	G	3505	T	C	C
2344	C	T	T	3540	C	C	T
2380	T	C	G	3621	T	C	C
2413	A	C	C	3654	C	T	T
				3681	T	C	C
2676	C	T	T	3702	A	G	G
2679	C	T	T	3720	C	A	A
2706	C	T	T	3849	T	C	C
2713	C	T	T	3873	G	A	A
2748	T	C	C	3954	A	G	G
2938	T	C	T	4209	C	T	T
2994	A	G	G	4365	A	G	G
2997	T	C	C	4434	C	T	T
3048	T	C	C	4506	C	T	T
3144	T	G	G	4602	T	C	C
3303	A	A	G	4614	A	G	G
3324	C	T	T	4662	T	A	A
3330	C	T	T	4728	T	C	C
3423	C	T	T	4833	G	G	A
3455	T	T	A	4938	G	A	G

a/ First five entries show changes that map in the gag portion.

and pSR-XD2. Culture supernatants collected daily, beginning 4 days post-transfection, were used to determine the titer of infectious viral particles. In the next 4 days the pSR-pol-beta-transfected cultures consistently contained 100-fold more infectious particles than the pSR-REP cultures (16). Replacement of the 2.5 kb EcoRI-KpnI pol fragment of SR-RSV with that of BH-RSV beta resulted in an enhancement of viral replication. Many functions have been attributed to the retrovirus pol gene, and we do not know which function in BH-RSV contributes to such a dramatic stimulation of infectious virus.

DISCUSSION

Two aspects of the work described above deserve brief comment: the nature of the BH-RSV alpha pol mutation as deduced from DNA and protein studies, and comparison of the pol se-

quences of BH-RSV alpha and beta with the sequence of PR-RSV.

Direct DNA sequence analysis of the BH-RSV alpha and beta pol genes has shown only one mutation giving rise to an amino acid difference between the mutant and the wild-type pol gene products. Protein studies indicated that although a full-length gag-pol precursor was synthesized in the BH-RSV alpha-infected cells, neither the mature, processed pol products nor any other polymerase related proteins were detected in BH-RSV alpha virions. In this respect, the pol defect in BH-RSV alpha seems to be quite different from defects of other known nonconditional pol mutants (reviewed in 24). BH-RSV alpha may be a packaging mutant with a single mutation in pol that renders the Pr180 protein unpackageable due to some conformational change(s). This putative conformational change(s) may prevent packaging in an indirect way. BH-RSV alpha virions do not contain a significant amount of tRNA^{trp} nor do they contain the other small RNAs found in avian retrovirus particles (25), thus implicating the reverse transcriptase protein in selection of tRNAs for packaging. Although there is no evidence that such selection and RNA-protein interaction are required for proper packaging, their absence could be responsible for generating the observed mutant phenotype.

Our experiments have shown that no mature pol proteins were detected in the infected cells, which would suggest, as earlier indicated (16,26,27), that Pr180 protein is packaged into the virion and then cleaved there. The processed pol proteins are not the initial substrates for packaging, and therefore the alpha lesion probably does not affect processing of the pol protein.

The protein analysis of BH-RSV alpha showing the physical absence of the polymerase was performed on several independent clones of nonproducer cells (12,16,22), whereas the restriction mapping and DNA sequence analysis were performed on one molecular clone. The possibility that the pBH-alpha clone represents one of many different mutations producing the same alpha phenotype (i.e., absence of the polymerase activity) cannot be excluded. Molecular analysis of different alpha mutants should be helpful in answering this question.

Comparison of the DNA sequence-deduced protein sequences of BH-RSV pol genes with the pol gene product of PR-RSV has shown that both alpha and beta pol proteins differ in 7 amino acids from the pol sequence of PR-RSV. In addition, the sequence of beta pol has a glutamine at the position corresponding to the nucleotides 3454-3456 (23), whereas BH-RSV alpha and PR-RSV pol proteins have leucine. It is possible that in the pol-positive beta strain this point mutation is able to phenotypically reverse the effect of one (or more) of the other mutations (relative to PR-RSV) found in both alpha and beta genes.

Studies examining the secondary and tertiary structure of the pol proteins could be helpful in the broader interpretation of the results reported here.

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