Characterization of vaccinia virus gene B12R

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We report the characterization of vaccinia virus gene B12R which is predicted to encode a 33K protein with 36% amino acid identity to the serine/threonine protein kinase encoded by vaccinia virus gene B1R. S1 nuclease protection experiments showed that gene B12R is transcribed early during infection from an initiation site 11 bp upstream of the open reading frame (ORF). The gene encodes a 33K polypeptide that is not required for virus replication in tissue culture nor for virus virulence in a murine intranasal model. Expression of the B12R gene in Escherichia coli produced an abundant 33K polypeptide which lacked protein kinase activity under conditions in which the protein kinases encoded by vaccinia virus gene B1R and African swine fever virus gene J9L are active.

Vaccinia virus, the prototype orthopoxvirus, is a large cytoplasmic virus. The dsDNA genome is predicted to encode more than 200 proteins many of which have amino acid sequence homology with cellular protein(s) (Goebel et al., 1990; Smith et al., 1991). Two genes, B1R and B12R, were predicted to encode related proteins (36% amino acid identity) with homology to protein kinases (Howard & Smith, 1989; Traktman et al., 1989). Recently, the B1R gene product was shown to be a serine/threonine protein kinase that is required for virus DNA replication, it is packaged into virions and phosphorylates ribosomal proteins in vitro (Banham & Smith, 1992; Lin et al., 1992; Rempel & Traktman, 1992; Banham et al., 1993). The B12R open reading frame (ORF) is poorly related to other protein kinase ORFs and lacks a recognizable ATP-binding site corresponding to conserved region I and the invariant aspartate and glycine residues in regions III and IV (Howard & Smith, 1989), which characterize the protein kinase family (Hanks et al., 1988). The deduced partial amino acid sequence of the corresponding gene in cowpox virus is identical to B12R except for one conservative amino acid substitution (Pickup et al., 1986; Howard & Smith, 1989). In this paper we have characterized the B12R gene and show that it is transcribed from a start site 11 bp upstream of the ORF early during infection and encodes a 33K protein. A deletion mutant (vAB1) lacking 83% of gene B12R (amino acid residues 42 to 277) replicates efficiently in cultured cells and is not attenuated in intranasally infected mice in comparison to the wild-type (wt) virus. The B12R protein expressed in Escherichia coli lacks protein kinase activity in vitro when assayed under conditions in which protein kinases of vaccinia virus and African swine fever virus (ASFV) are active (Banham & Smith, 1992; Baylis et al., 1993).

Northern blotting had established previously that the B12R gene was transcribed early during infection forming a major transcript of 1100 nucleotides (nt) (Howard & Smith, 1989). To locate the 5' initiation site accurately, RNA was extracted from vaccinia virus-infected cells early or late during infection (Smith et al., 1989b) and hybridized with a 510bp EcoRV-BglII DNA fragment radiolabelled with 32P at the BglII site 255 bp downstream of the 5' end of the B12R ORF. A 266 bp fragment was protected from S1 nuclease digestion by early but not late RNA (Fig. 1a). This placed the major 5' end of the RNA at position −11 relative to the start of the ORF with minor transcripts initiating at positions −12, −13 and −15 (Fig. 1b). These data are consistent with the observed 1100 nt early RNA (Howard & Smith, 1989) and the presence of the early transcription termination signal TTTTNT (Yuen & Moss, 1987) 11 to 20 nt downstream of the 3' end of the ORF, and also established that the entire B12R ORF, which had been predicted by computer, was likely to be expressed during virus infection.

To determine whether the B12R protein had protein kinase activity, the gene was engineered for expression in E. coli using a strategy similar to that previously employed for the vaccinia virus B1R ORF (Banham & Smith, 1992). Plasmid pSTH1 (Howard, 1991) was

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digested with *Cl*<sub>AI</sub> and a 978 bp fragment containing *B12R* was treated with the Klenow enzyme and ligated into *Sma*<sub>1</sub>-digested *pUC119* so that the *EcoRI* site of the polylinker was upstream of the *B12R* ORF. This plasmid was called *pAB13*. *EcoRI* and *NdeI* restriction sites were engineered adjacent to the 5' end of the *B12R* gene by digesting *pAB13* with *EcoRI* and *Bgl*<sub>II</sub>, and replacing this fragment by an *EcoRI*–*Bgl*<sub>II</sub> DNA fragment that had been generated by PCR using oligonucleotides 5' CCGGAATTCATATGGGAATCTCCCTCAAGT and 5' AATTCCATACAGATCTGGAA and plasmid *pAB9* (described below) as the template. The resultant plasmid, *pAB15*, was sequenced to check the authenticity of the PCR fragment. Finally, the *B12R* ORF was excised from *pAB15* as a 0.98 kb *NdeI*–*HindIII* DNA fragment and transferred into vector *pGMT7* [a derivative of the bacteriophage T7 expression vector *pET-3c* (Rosenberg *et al.*, 1987) containing a multiple cloning site polylinker immediately downstream of the T7 gene 10 translational start site] which had been digested with the same enzymes. The resultant plasmid, *pAB19*, contained the *B12R* ORF positioned downstream of the T7 promoter and with its ATG initiation codon close to the prokaryotic ribosome binding site in *pGMT7*. This plasmid was transformed into *E. coli* strain BL21 (DE3)/pLysS (a λ lysogen containing gene 1 of bacteriophage T7, encoding RNA polymerase, under the control of the *lac* UV5 promoter) and expression of the *B12R* ORF was induced with 1 mM-IPTG. Fig. 2(a) shows that a 33K (*B12R*) protein accumulated to high levels by 3-5 h post-induction (lane 3) but was absent prior to induction (lane 2) and from induced cells containing the parental vector *pGMT7* (lane 1). After 3.5 h induction the *B12R* gene product was largely insoluble and formed inclusion bodies (lane 4). For biochemical analysis of the *B12R* product, soluble non-denatured protein was required. The amount of *B12R* protein in the soluble extracts of infected cells was, therefore, increased by induction with a lower concentration of IPTG (100 μM), and harvesting the cells after a shorter induction period (2 h) (data not shown).

Extracts from these cells were prepared and assayed under conditions in which both the vaccinia virus B1R gene product and the ASFV j9L protein expressed in *E. coli* had protein kinase activity (Banham & Smith, 1992; Baylis *et al.*, 1993) (Fig. 2b). Although *B1R* phosphorylates both casein and histones under these conditions *in vitro* (Fig. 2b, lanes 5 and 6), *B12R* did not exhibit greater activity with these substrates (lanes 3 and 4) than extracts from cells containing *pGMT7* (lanes 1 and 2). Similar results were obtained when BSA, phosphorylase or protamine were incubated with extracts containing *B12R* (data not shown) and when the *B12R* protein was assayed immediately after preparation rather than after freezing and thawing. In summary, under the conditions used, the *B12R* protein did not exhibit protein kinase activity, whereas two other virus protein kinases were active despite having different amino acid and protein substrate specificities.

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A New Zealand White rabbit was immunized with 200 µg of a B12R inclusion body preparation in complete Freund's adjuvant and then re-immunized seven times over a 10 month period with 250 µg of the inclusion body protein in incomplete Freund's adjuvant. The protein used for immunization was a 29K truncated form of BI2R produced from plasmid pAB4 in the above expression system and thought to result from translational initiation within the B12R ORF. Plasmid pAB4 was formed by digestion of pSTH1 with Clal, treatment with the Klenow enzyme and ligation of a 978 bp fragment into pGMT7 that had been digested with NdeI and treated with the Klenow enzyme. The immune sera were used to identify the B12R protein within vaccinia virus-infected cells (Fig. 2c). CV-1 cells were infected at 50 p.f.u./cell with either wt virus or a deletion mutant, vAB1, lacking the B12R gene (see below) in the presence or absence of cytosine arabinoside (AraC), an inhibitor of DNA replication. Infected cells were pulse-labelled with trans-[35S] (ICN Flow) and extracts were prepared and immunoprecipitated with anti-B12R serum as previously described (Banham & Smith, 1992). A protein of 33K was immunoprecipitated from wt virus-infected cells (lane 4) an was also synthesized in the absence of DNA replication (lane 5), consistent with the transcriptional data. In contrast, the protein was not detected in cells infected with vAB1 (lanes 2 and 3), nor in mock-infected cells (lane 1). It was possible that the protein slightly larger than B12R which was present in lanes 2 to 5 might have been B1R that was cross-reactive with the anti-B12R serum. This was addressed by immuno-

Fig. 2. Analysis of the B12R protein. (a) Bacterial expression of the B12R gene in E. coli BL21 (DE3) LysS cells containing pGMT7 (lane 1) or pAB19 (lanes 2 and 3) were induced with 1 mM IPTG for 3.5 h (lanes 1 and 3) or not induced (lane 2). Cell aliquots were pelleted, resuspended in protein sample buffer and visualized by staining with Coomassie blue after SDS-PAGE on a 10% gel. Lane 4 shows an equivalent amount of insoluble inclusion body prepared in parallel with sample 3. The sizes of protein M, standards are shown (lane M). The position of B12R is indicated with an arrow. (b) Assay for protein kinase activity. Ten µg of either casein (lanes 1, 3 and 5) or calf thymus histones (lanes 2, 4 and 6) were incubated with extracts from E. coli cells expressing B12R from plasmid pAB19 (lanes 3 and 4), B1R from plasmid pAB6 (Banham & Smith, 1992) (lanes 5 and 6) or containing the parental vector pGMT7 (lanes 1 and 2). Proteins were resolved by SDS-PAGE on a 15% gel and visualized by autoradiography. The position of migration of 14C-labelled M, standards are shown (lane M). (c) Immunoprecipitation of B12R from vaccinia virus-infected cells. CV-1 cells were infected with either WR (lanes 4 and 5), vAB1 (lanes 2 and 3) or mock-infected (lane 1) in the presence (lanes 2 and 4) or absence (lanes 1, 3 and 5) of 40 µg/ml AraC. Proteins were labelled with trans-[35S] 2 to 4 h p.i. and extracts were prepared and immunoprecipitated with anti-B12R serum as described (Banham & Smith, 1992). Immune complexes were analysed by SDS-PAGE on a 10% gel and autoradiography. M, standards are shown (lane M).
was transcribed in the same direction as vaccinia virus strain WR, transfected with pAB10 and virus was titrated on duplicate monolayers of BSC-1 cells (Mackett et al., 1985). Cells were frozen and thawed three times, sonicated and intracellular medium. At various times p.i. the cells were scraped into the medium, pelleted by centrifugation and resuspended in 1 ml MEM (Gibco). Cells were infected with wt vaccinia virus WR in cell culture or in intranasally infected mice. The protein did not possess protein kinase activity when expressed in E. coli (Banham & Smith, 1992; Baylis et al., 1993), although two other virus protein kinases were active under conditions in vivo which two other virus protein kinases were active (Banham & Smith, 1992; Baylis et al., 1993), although the detergents used in the extraction procedure may have dissociated such complexes.

To assess the role of the B12R gene in the vaccinia virus life cycle, a deletion mutant lacking the gene was constructed by the transient dominant selection method (Falkner & Moss, 1990). A 1.95 kb HindIII DNA fragment was excised from pSTH1 and ligated into Smal-digested pSJJH7 (Hughes et al., 1991) so that B12R was transcribed in the same direction as vaccinia virus 7.5K promoter driving the E. coli guanine phosphoribosyltransferase (Ecogpt) selectable marker (Boyle & Coupar, 1988). This plasmid, pAB9, was digested with SnaBI to remove a 703 bp internal fragment comprising 83 % of the B12R ORF (amino acids 42 to 277) and then religated to form pAB10. Cells were infected with wt vaccinia virus strain WR, transfected with pAB10 and then mycophenolic acid (MPA)-resistant viruses were isolated (Boyle & Coupar, 1988). These MPA-resistant viruses containing the integrated sequences of plasmid pAB10 were then plated onto D98R cells (Kerr & Smith, 1991) in the presence of 6-thioguanine (6-TG) (Isaacs et al., 1990) and 6-TG-resistant isolates, which had lost the plasmid sequences, were plaque-purified. The genome structure of the recombinant virus was confirmed by Southern blotting (data not shown). The isolation of the deletion mutant, vAB1, demonstrated that the B12R ORF was non-essential for replication of vaccinia virus strain WR in cell culture and is consistent with the deletion of this gene from vaccinia virus strain Copenhagen without loss of infectivity (Perkus et al., 1991). To determine whether there were subtle differences in replication rate between the wt virus and deletion mutant, each virus was used to infect BSC-1 cells at low multiplicity and the kinetics of intracellular virus production were determined (Fig. 3). The rate of virus production and the final titre obtained were indistinguishable for the two viruses.

To evaluate any contribution of the B12R ORF to virus virulence in vivo we compared the virulence of wt virus and the deletion mutant vAB1, in groups of eight intranasally infected, female, 5- to 6-week-old, BALB/c mice at virus doses of 10^4, 10^5 and 10^6 p.f.u. All animals infected with virus doses of 10^6 or 10^7 and which had survived to day 8 post-infection (p.i.) were then sacrificed as their mean body weights had reached > 70 % of that prior to infection. In our experience with this model this represents a reasonable humane endpoint. Animals infected with either virus at a dose of 10^4 p.f.u. survived infection and had a similar maximum weight loss (25 % for wt virus and 21 % for vAB1) by day 8. By day 19 the weights of these groups had recovered to 92 % and 95 % of original weights, respectively. Overall these data showed that the virulence of the two viruses was indistinguishable. A second experiment measuring mortalities with a group size of 10 gave a similar conclusion (data not shown).

In summary, the B12R gene has been shown to encode a 33K protein that is expressed early during virus infection and which is not required for the replication of vaccinia virus WR in cell culture or in intranasally infected mice. The protein did not possess protein kinase activity when expressed in E. coli under conditions in which two other virus protein kinases were active (Banham & Smith, 1992; Baylis et al., 1993), although the possibility of B12R phosphorylating a more natural substrate in vivo cannot be ruled out. The lack of kinase activity was not unexpected, as although the protein shares 36 % amino acid identity with the active B1R protein kinase, it is divergent from protein kinases in several conserved amino acid residues (see above).
Additionally, the deletion of each non-essential vaccinia virus gene encoding an active enzyme has correlated with a reduction in virus virulence, for example thymidine kinase (Buller et al., 1985), ribonucleotide reductase (Child et al., 1990), DNA ligase (Kerr et al., 1991), thymidylate kinase (G. L. Smith, unpublished data) and a steroid dehydrogenase (Moore & Smith, 1992). The lack of attenuation of virus vAB1 is, therefore, consistent with the lack of enzyme activity. Some other vaccinia virus genes that appear not to have altered virus virulence in this model include the serpin K2L (Law & Smith, 1991), which prevents virus-induced cell fusion. The function of the B12R protein remains unknown.

The homology of B12R with B1R suggests that these genes have derived from a common ancestor by an ancient gene duplication event. Gene families have been found in several large DNA viruses including vaccinia virus (Smith et al., 1991), Shope fibroma virus (Upton et al., 1987), fowlpox virus (Tomley et al., 1988), ASFV (Almendral et al., 1990) and human cytomegalovirus (Weston & Barrell, 1986). Thus it is not uncommon for large DNA viruses to have increased their genome complexity by apparent gene duplication and functional divergence.

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References


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