Rubella Virus cDNA

SEQUENCE AND EXPRESSION OF E1 ENVELOPE PROTEIN*

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A cDNA clone encoding the entire E1 envelope protein (410 amino acid residues) and a portion of the C-terminal end of the E2 envelope protein of the rubella virus has been isolated and characterized. DNA sequence analysis has revealed a region 20 nucleotides in length at the 3′ end of the cloned cDNA which may be a replicase recognition site or a recognition site for encapsidation. The proteolytic cleavage site between the E1 and E2 proteins was localized based on the known amino-terminal sequence of the isolated E1 protein (Kalkkinen, N., Oker-Blom, C., and Pettersson, R. F. (1984) J. Gen. Virol. 65, 1549–1557) and the deduced amino acid sequence. The mature E1 protein is preceded by a set of 20 highly hydrophobic amino acid residues possessing characteristics of a signal peptide. This "signal peptide" is flanked on both sides by typical protease cleavage sites for trypsin-like enzyme and signal peptidase. The presence of a leader sequence in E1 may facilitate its translocation through the host cell membrane.

The E1 protein of rubella virus shows no significant homology with alphavirus E1 envelope proteins. However, a stretch of 39 amino acids in the E1 protein of rubella virus (residues 262–300) was found to share a significant homology with the first 39 residues of bovine sperm histone. The position of 4 half-cystines and 8 arginines overlaps.

The E1 protein of rubella virus has been successfully expressed in COS cells after transfecting them with rubella virus cDNA in simian virus 40-derived expression vector. This protein is antigenically similar to the one expressed by cells infected with rubella virus.

Rubella was first described in the 18th century in Germany, and thus, the name German measles was coined. Rubella in children and young adults is characterized by rash and mild fever. During early pregnancy, however, rubella virus infection can cause fetal death or multisystem birth defects including deafness, cataracts, mental retardation, and congenital heart disease (1–6). An essential breakthrough in the development of the rubella virus has been achieved in 1962 with the isolation of the rubella virus in cell culture by Parkman et al. (7) and by Weller and Neva (8). A worldwide epidemic of rubella in 1963–1965 prompted the development of an effective vaccine in 1966 by Parkman et al. (9) and other groups (10, 11). As a result of vaccination, there has been a dramatic reduction in the incidence of rubella and congenital rubella in children of early age groups; however, the majority of cases with rubella are now found in older adolescents and young adults.

The structure of the virus and the mechanism by which the virus infects the cell have been the subject of many studies (12, 13). Several groups of investigators have tried to elucidate the structure of the rubella virus by characterizing the viral proteins (14–18). The general consensus reached by these groups is that the virus consists of a nucleocapsid protein (M, = 30,000) and three envelope glycoproteins, E1 (M, = 58,000), E2a (M, = 47,000), and E3 (M, = 42,000) (17, 18). However, tryptic peptide analysis has indicated that E2a and E3 are closely related and may represent two different glycosylated forms of the same polypeptide (17). It has been shown that the genome of rubella virus consists of a 40 S single-stranded polyadenylated RNA, which is infectious (19–22). In infected cells, a subgenomic 24 S RNA which is derived from the 3′ end of the 40 S RNA has been identified (22). This 24 S RNA encodes a polypeptide of M, 110,000 which is thought to be the precursor for the envelope and capsid proteins (22).

As part of our plan to investigate the mechanism of viral infection of host cells and to devise possible virus-specific therapeutic agents, we have undertaken the isolation and characterization of the rubella virus genome. In this report, we summarize our findings on the elucidation of the structure of a cDNA clone encoding the E1 protein of rubella virus.

EXPERIMENTAL PROCEDURES†

RESULTS

In Vivo and in Vitro Synthesis of Rubella Virus Structural Proteins—Three radioactive bands corresponding to molecular masses of 58, 42, and 30 kDa were visualized upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunoprecipitated extracts of [35S]methionine-labeled virus-infected cells but not from the control cells (data not shown). In the presence of tunicamycin, the intensity of the 58- and the 42-kDa proteins decreased with the concomitant appearance of two new radioactive protein bands at positions corresponding to 43 and 35 kDa (data not shown), thereby indicating that the 58- and the 42-kDa proteins are likely to be glycosylated.

† Portions of this paper (including "Experimental Procedures" and Figs. 2, 5, and 7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J02620.
In vitro translation of poly(A)* RNA extracted from virus-infected cells in the rabbit reticulocyte system showed a polypeptide of 110 kDa precipitable with anti-rubella virus antibodies. Poly(A)* RNA prepared from uninfected cells failed to translate this protein (data not shown). These experiments suggest that infection of the Vero 76 cells with rubella virus induces the production of a protein of 110 kDa which presumably could be post-translationally processed to yield the 58-, 42-, and 30-kDa proteins.

Cloning of Rubella Virus mRNA—cDNA libraries from virus-infected cultures were grown on duplicate filters and screened for rubella virus mRNA using cDNAs synthesized from either infected or uninfected cell total poly(A)* RNA. One set of filters was hybridized to α-32P-labeled cDNA synthesized from infected cell total poly(A)* RNA, and the second set was hybridized to α-32P-labeled cDNA synthesized from control cell total poly(A)* RNA. Approximately 110 cDNA clones were obtained which hybridized preferentially to the infected cDNA probe. A cDNA clone (pRv45a) with the longest insert, 1889 bp, was isolated. To confirm that the pRv15 cDNA clone was complementary to rubella virus RNA, a 32P-labeled PstI insert from clone pRv45a was hybridized to total and poly(A)* RNAs isolated from uninfected cells, infected cells, and viral particles. Fig. 1 shows the Northern blot analysis of such RNAs. Lanes 1 and 2 from uninfected cells do not show hybridization. Lanes 3 and 4 are from infected cells and show three hybridizable bands of 10.2, 9.5, and 1.7 kb. Lane 5 is RNA isolated from viral particles and shows one hybridizable band of 10.2 kb. The sizes of RNAs identified are in agreement with the published reports for rubella virus genomic and subgenomic RNAs except for the additional 1.7-kb RNA (22). The presence of the additional 1.7-kb RNA could be due to an as yet unidentified subgenomic RNA species or to a defective intermediate RNAs.

Nucleotide Sequence of pRv45 DNA—The restriction map and the sequencing strategy of pRv45 are described in Fig. 2. The nucleotide sequence was also determined from another cDNA clone (pRv45) which is 1500 bp long and has only one PstI insert as compared to two PstI inserts found in pRV45. The nucleotide sequence was determined in both directions by the method of Maxam and Gilbert (29). The complete nucleotide sequence of pRV45 is given in Fig. 3. The cDNA clone is 1822 bp long and has a poly(A)* tail of 17 residues at the C-terminal end and a C-tail of 25 residues on each end. One long open reading frame of 522 amino acid residues was revealed with two in-phase termination codons. The first termination codon is followed by a 253-nucleotide long 3'-noncoding region. No putative polyadenylation signal sequence was found upstream of the polyadenylation site. Comparison of the amino acid sequence with the sequences in the protein data bank showed that a stretch of 39 amino acids between residues 262 and 300 showed similarity with bovine sperm histone (Fig. 4).

Construction of Rubella Virus E1 Protein Expression Plasmid pcDpLRv45.—The scheme for the construction of pcDpLRv45 plasmid is shown in Fig. 5. The chimeric plasmid was constructed by introducing a PstI fragment (1500 bp) of rubella virus cDNA (pRV45) which codes for E1 protein into the PstI site of the pL vector adjacent to the SV40 early promoter. From this construct, a HindIII/EcoRI fragment which contains the SV40 early promoter and the rubella virus cDNA were excised and ligated to a HindIII/EcoRI fragment of the pcD vector. The HindIII/EcoRI fragment of the pcD vector contains an ampicillin-resistant gene and SV40 polyadenylation signal sequences. This chimeric plasmid designated pcDpLRv45 contains the SV40 early promoter, rubella virus cDNA, and SV40 polyadenylation signal in that order.

Expression of E1 Glycoprotein in COS Cells—Expression of rubella virus cDNA coding for E1 protein was measured both by the production of rubella virus mRNA and protein after transfection of pcDpLRv45 DNA into COS cells (Fig. 6). Cells were labeled with [35S]methionine for 2 h after 72 h transfection. Total proteins were extracted and immunoprecipitated using rubella virus antibody. A protein of 58 kDa was observed in cells transfected with pcDpLRv45 DNA (Fig. 6A, lane 6). This protein migrated with E1 protein which was immunoprecipitated from the rubella virus-infected Vero 76 cells (Fig. 6A, lane 7). No such protein band was precipitated from COS cells, COS cells transfected with either the pcD vector or pcD-dhfr plasmid (Fig. 6A, lanes 2–5). Lack of similar protein band was observed in uninfected cells as well (Fig. 6A, lane 8).

Total RNA was isolated 72 h after transfection, and rubella virus mRNA was detected and characterized by the S1 nuclease procedure using a 3'-end-labeled DNA probe (Fig. 6B). PstI insert (1500 bp long) of the rubella virus cDNA pRV45 which encodes the entire E1 protein was labeled at the 3'-protruding end with [α-32P]dideoxy-ATP using terminal deoxynucleotidyltransferase enzyme. The labeled fragment was used in hybridization reaction with various RNAs and then subjected to S1 nuclease digestion. Hybridization with RNA isolated from the COS cells transfected with pcDpLRv45 DNA protected the 1500-bp fragment (Fig. 6B, lane 4). A similar size fragment was also protected by RNA from rubella virus-infected cells (Fig. 6B, lane 5). However, this fragment was not protected when RNA either from COS cells transfected with pcD vector (Fig. 6B, lane 3) or from uninfected cells (Fig. 6B, lane 6) was hybridized. The labeled fragment in the absence of RNA was completely digested with S1 nuclease (Fig. 6B, lane 2). Thus, from the protein as well as RNA data, it is evident that E1 glycoprotein of rubella virus is expressed in COS cells from the pcDpLRv45 DNA under the control of SV40 promoter. The protein synthesized in these cells is antigenically similar to the one expressed in viral infected cells. In addition, these data further confirm the identity of the pRV45 cDNA clone.
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Fig. 4. Comparison of rubella virus E1 glycoprotein and bovine sperm histone. Amino acids are designated by single-letter code. Homologous regions are boxed. Solid boxes represent same amino acid, where as dotted boxes represent conservative changes. ReE1, rubella virus E1 glycoprotein; BS8, bovine sperm histone. The numbers represent the position of the amino acids.

Fig. 6. Expression of rubella virus E1 glycoprotein cDNA in cos cells. A, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of [35S]methionine-labeled proteins from transfected COS cells. Arrows designate the 14C-labeled protein markers. From the top, the sizes of markers are 92, 68, 43, 25, 18, and 14 kDa (lane 1). Lanes 2–8 are immunoprecipitates of labeled proteins: lane 2, from nontransfected COS cells; lane 3, mock transfected COS cells; lane 4, pcD vector-transfected COS cells; lane 5, pcD-dhfr-transfected COS cells; lane 6, pcDpLRv4-transfected COS cells; lane 7, rubella virus-infected Vero 76 cells; lane 8, uninfected Vero 76 cells. B, S1 nuclease analysis. Lane 1, Psfl fragment labeled with [α-32P]dideoxy ATP without S1 nuclease treatment; lane 2, with S1 nuclease; lane 3, hybridized with RNA isolated from pcD vector-transfected COS cells and digested with S1 nuclease; lane 4, hybridized with RNA isolated from pcDpLRv4-transfected COS cells and digested with S1 nuclease; lane 5, hybridized with RNA isolated from rubella virus-infected Vero 76 cells and digested with S1 nuclease; lane 6, hybridized with RNA isolated from uninfected cell RNA and digested with S1 nuclease. The molecular mass markers depicted are derived from HindIII-digested λ DNA and HaellI-digested φX174 replicative form DNA.

DISCUSSION

A cDNA clone coding for the entire E1 glycoprotein and a C-terminal portion of the E2 protein of the rubella virus has been isolated and characterized. The cDNA clone isolated hybridized to two size classes of RNAs (10.2 and 3.5 kb) isolated from virus-infected cells and one size class of RNA (10.2 kb) from purified virus particles (Fig. 1), confirming that the cDNA clone isolated is rubella virus-specific. Similar sizes of RNA for rubella virus have been identified by other investigators (19–22). In addition, the isolated cDNA clone

Fig. 3. Nucleotide sequence of rubella virus 24 S RNA and amino acid sequence of encoded proteins. The coding region is denoted by the nucleotide sequence in triplets. The termination codons are noted by asterisks. The arrows pointing up or down show the probable protease cleavage sites. The start of E1 glycoprotein is designated by an arrow followed by E1. The underlined amino acids are the same as those derived from amino acid sequence analyses of E1 glycoprotein. The putative glycosylation sites are indicated (•).
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hybridized to a 1.7-kb RNA species from infected cells. Whether this RNA represents another subgenomic viral RNA or a defective interfering RNA is not known at this time. Defective interfering RNAs approximately 2 kb in size have been isolated and characterized from both Sindbis virus and Semliki forest virus (37-39). The 3′-noncoding region of the rubella virus cDNA clone lacks the consensus sequence for the polyadenylation signal. Similar lack of polyadenylation could be a transmembrane region. Similar transmembrane regions are found in alphaviruses (40, 41). The overall hydropathy profile of rubella virus E1 protein is similar to that of E1 proteins from alphaviruses even though they do not share significant homology in their protein structures.

It is tempting to speculate that the E1 protein is first synthesized as a precursor and then processed by proteolytic cleavage at either Arg235 or Arg270 in the polyprotein as shown in Fig. 3. The precursor form of E1, with the hydrophobic "signal" peptide would be further processed by the host protease asp/at to form a mature E1 protein upon secretion. Similar hydrophobic sequences have been found between E1 and E2 proteins of Sindbis and Semliki forest viruses (40, 41). The presence of hydrophobic sequences in the E1 protein of alphaviruses may facilitate the translocation of these proteins through host cell membranes.

The E1 protein consists of a single polypeptide chain with 410 amino acids. The molecular weight deduced from amino acid composition is about 43,000, which agrees with the non-glycosylated form of E1 protein. The difference between the glycosylated form of E1 (M, 58,000) and the nonglycosylated E1 (M, 43,000) represents the amount of carbohydrate attached to the protein. There are three putative N-glycosylation sites, Asn-X-Thr/Ser, in the E1 protein at residues 76, 177, and 209 (Fig. 3). The nature of the carbohydrate and the actual site of glycosylation are not known at this time.

The deduced amino acid sequence of the E1 protein from rubella virus shows no significant homology with all other proteins for which structures have been established, including the alphaviruses. However, a stretch of 39 amino acids (residues 262-300) in the E1 protein of rubella was found to share a significant sequence homology with residues 1-39 of a 47-amino acid residue bovine sperm histone (Fig. 4). The similarity is found in the arrangement of half-cystine and arginine residues which appear to be conserved in these segments of the two proteins (54). The position of 4 half-cystines and 8 arginines overlaps (Fig. 4). In the bovine sperm histone, the half-cystines are believed to be involved in disulfide bond formation to provide stability for optimal interaction of the positively charged arginine functional groups with deoxyribonucleic acids.

DNA sequence analysis of clone pPR4 shows a long open reading frame coding for 522 amino acids. Based on the previously established amino acid sequences of a 12-amino acid fragment in the amino-terminal portion of the purified E1 protein from the Thérien strain of rubella virus (46), the starting site of the deduced E1 protein from the cDNA clone has been assigned to the glutamic acid residue as shown in Fig. 3. This residue is preceded by a highly hydrophobic consensus sequence. The presence of hydrophobic region of residues 1 to 20 is flanked on both sides by potential protease cleavage sites. Ala-Glu-Lys, a typical cleavage site for signal peptidase (47), and Cys-Ala-Cys-Arg-Arg-Ala-Cys-Arg-Arg, containing double basic amino acids, is typical of a trypsin-like enzyme cleavage site (48-52). The upstream amino acid sequence from residue 20 to 112 could be part of the E1 envelope protein since the gene order of structural proteins in rubella virus has been shown to be NH2-terminal capsid, E1 glycoprotein, E2 glycoprotein, and carboxyl terminus (53).

It is tempting to speculate that the E1 protein is first synthesized as a precursor and then processed by proteolytic cleavage at either Arg235 or Arg270 in the polyprotein as shown in Fig. 3. The precursor form of E1, with the hydrophobic "signal" peptide would be further processed by the host protease at.

The E1 protein of rubella virus has been successfully expressed in COS cells using a SV40-derived expression vector system. The E1 protein produced is antigenically similar to the one expressed by cells infected with rubella virus in that it could be immunoprecipitated by anti-rubella antibody. Whether antibodies developed against the E1 protein could neutralize rubella virus remains to be established. The rubella virus cDNA clone isolated and characterized here might be useful as a diagnostic probe for the detection of viral sequences in clinical situations especially with pregnant women.

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Rubella Virus cDNA: Sequence and Expression of Envelope Proteins

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Experimental Procedures

Fig. 5. mRNA Labeling and Autoradiography

The reaction mixture was incubated at 37°C for 20 minutes. The labeled fragment was separated by electrophoresis on a 1% agarose gel. The gels were autoradiographed at -70°C overnight.

Fig. 6. Scheme for the construction of rubella virus cDNA clones. The direction and extent of each fragment is indicated by the direction of arrow, originating sequencing at the restriction site shown. The vertical arrows show the sites for restriction endonucleases present in the polylinker. The DNA sequence shown is the one derived from the restriction enzyme digestion of the cDNA clone at the end of the insert. The insert was labeled with [α-32P]dCTP by nick translation as described above and used as a probe for hybridization. The sequence of the insert was determined by the dideoxy chain-termination method using Sequenase (version 2.0) according to the manufacturer's instructions. The sequences obtained were then compared with those of the rubella virus genome sequence.