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Complementation Analysis of the Dales Collection of Vaccinia Virus Temperature-Sensitive Mutants

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A collection of randomly generated temperature-sensitive (ts) vaccinia virus (strain IHD-W) mutants were reported by S. Dales *et al.,* (1978, *Virology*, 84, 403–428) in 1978 and characterized by electron microscopy. We have performed further genetic analysis on the Dales collection of mutants to make the mutants more useful to the scientific community. We obtained the entire Dales collection, 97 mutants, from the American Type Culture Center (ATCC). All 97 mutants were grown and reassessed for temperature sensitivity. Of these, 16 mutants were either very leaky or showed unacceptably high reversion indices even after plaque purification and therefore were not used for further analysis. The remaining 81 ts mutants were used to perform a complete complementation analysis with each other and the existing Condit collection of ts vaccinia virus (strain WR) mutants. Twenty-two of these 81 Dales mutants were dropped during complementation analysis due to erratic or weak behavior in the complementation test. Of the 59 mutants that were fit for further investigation, 30 fall into 13 of Condit's existing complementation groups, 5 comprise 3 previously identified complementation groups independent of the Condit collection, and 24 comprise 18 new complementation groups. The 59 mutants which were successfully characterized by complementation will be accessioned by and made available to the scientific community through the ATCC.

Key Words: temperature-sensitive; complementation; IHD-W; genetics; vaccinia.

INTRODUCTION

Poxviruses are large double-stranded DNA viruses that replicate in the cytoplasm of host cells. In an effort to understand the replication of these viruses, several groups have isolated randomly generated, temperature-sensitive (ts) virus mutants (Padgett and Tomkins, 1968; Dales *et al.,* 1978; Chernos *et al.,* 1978; Condit and Motyczka, 1981; Ensinger, 1982; Condit *et al.,* 1983; Drillien and Spehner, 1983; reviewed in Condit and Niles, 1990). Many of these conditional–lethal mutants were sorted into complementation groups and assigned to physical map positions using marker rescue. Some, for example the Condit collection, have been systematically subjected not only to complementation and physical mapping analysis, but to phenotypic analysis as well. The Condit collection comprises 63 mutants in 32 complementation groups of which 79% have been mapped to individual genes. Phenotypic analysis of this collection, based on protein synthesis and DNA replication, revealed four biochemical phenotypes. Mutants that were indistinguishable from wt with respect to protein and viral DNA synthesis at the permissive and nonpermissive temperatures characterized the "normal" phenotype. Mutants that synthesized no viral DNA or late viral proteins at the nonpermissive temperature characterized the "DNA-negative" phenotype. Mutants that showed normal DNA synthesis and weak or delayed late viral protein synthesis at the nonpermissive temperature characterized the "defective late" phenotype. Finally, the "abortive late" phenotype was characterized by normal DNA synthesis at the nonpermissive temperature and late viral protein synthesis that began normally at the nonpermissive temperature and then ceased abruptly (Condit and Motyczka, 1981). The complete complementation and partial physical mapping of the Condit collection has made these mutants extremely useful and created a major resource for vaccinia mutants.

Systematic genetic analysis of some other collections has been more limited. For example, the Dales collection of approximately 90 mutants was characterized and grouped into 17 categories, A to Q, based on the phenotypic defects shown by electron microscopy (EM) at the nonpermissive temperature (Table 1) (Dales *et al.,* 1978). These categories represent, in order of increasing complexity, the stages of viral assembly reached during infection at the nonpermissive temperature. However, in the absence of complementation analysis, it is unclear whether mutants possessing similar or different EM phenotypes represent mutations in the same or different genes. Nevertheless, the Dales collection is a rich resource of mutants. For example, it comprises a fascinat-

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TABLE 1

Categories of Morphological Aberrations

Category	Description
A	No virus structure evident
B	Foci of viroplasm
C	Rudimentary virus membranes with spicules in foci of viroplasm
D	Foci of viroplasm and DNA paracrystals
E	Foci of viroplasm, aberrant membranes without spicules, and DNA paracrystals
F	Aberrant membranes with spicules and viroplasm
G	Incomplete membranes with spicules
H	Composite of D–F as well as complete immature particles
I	Similar to G as well as complete immature particles
J	Composite of E, F, and I as well as dispersed immature particles
K	Granular foci and immature particles with nucleoids but lacking internal dense material
L	Immature particles with nucleoids and defective membranes with spicules
M	Immature particles without nucleoids and intermediate maturing forms
N	Factories containing immature and numerous intermediate maturing forms
O	Immature normal particles and mature particles with aberrant cores
P	Immature normal particles and mature particles with cores that are predominantly lucent at the center
Q	Immature and mature particles of normal appearance

Note. Table adapted from Dales *et al.* (1978).

ing array of EM phenotypes (Szajner *et al.,* 2001), and it contains the only known ts mutations in the vaccinia virus uracil glycosylase and in a subunit of the mRNA capping enzyme (Carpenter and DeLange, 1991; Millns *et al.,* 1994). We therefore felt it would be of value to not only conduct complementation analysis with the Dales group but to also test the Dales mutants against the Condit complementation groups to integrate the two collections.

We have obtained the entire Dales collection of 97 vaccinia virus (strain IHD-W) mutants from the ATCC and undertaken complementation analysis of this collection to organize and categorize the mutants. The collection was first verified for temperature sensitivity, allowing us to identify those mutants that would work well in complementation analysis. Eighty-one mutants were selected for complementation analysis with each other and with the existing Condit collection of ts vaccinia virus (strain WR) mutants. Complementation analysis eliminated 22 additional viruses due to erratic or weak complementation and revealed 18 new complementation groups in addition to the 35 previously described by Condit *et al.* (1983); Szajner *et al.* (2001), Millns *et al.* (1994), and Carpenter and DeLange (1991). This analysis provides a renewed source of useful ts mutants and increases the number of genes covered. The purpose of this article is to summarize the complementation analysis and compile an accurate overview of ts mutants in vaccinia virus from several collections including mutants isolated by Condit and co-workers, some of the mutants isolated by Ensinger and co-workers, and the mutants isolated by Dales and co-workers (Dales *et al.,* 1978; Condit and Motyczka, 1981; Ensinger, 1982; Condit *et al.,* 1983; Seto *et al.,* 1987). The assignment of the Dales mutants to the Condit complementation groups is discussed and a description of each of the Dales mutants currently available through the ATCC is presented.

RESULTS AND DISCUSSION

Dales *et al.* (1978) originally grew and plaqued vaccinia IHD-W virus in L_2 cells, a substrain of mouse L_{929} fibroblasts. Subsequently, all of the ts mutants were isolated and characterized in this cell line as well. These cells, as described by Dales *et al.,* were not available from ATCC. (The existing ATCC L_2 cells are designated as rat epithelial lung cells.) The Condit collection, consisting of ts mutants in the vaccinia WR strain, were isolated and characterized in African green monkey kidney BSC40 cells (Condit and Motyczka, 1981). We therefore performed a preliminary experiment to compare the growth and phenotype of the vaccinia IHD-W and WR strains in BSC40 and L_{929} cells. The wild-type vaccinia WR and IHD-W strains were grown in both BSC40 and L_{929} cells at 37°C and were subsequently plaqued in both BSC40 and L_{929} cells at 31, 37, and 39.5°C. Vaccinia strain WR grew and plaqued better in BSC40 cells than in L_{929} cells. The IHD-W strain grew and plaqued equivalently in BSC40 and L_{929} cells; however, the plaques were larger on BSC40 cells (data not shown). In general, the BSC40 cells were easier to work with than L_{929} cells; they grew faster and formed tighter monolayers. As a result of these tests, we chose to perform all subsequent experiments with BSC40 cells exclusively.

Dales *et al.* (1978) used a four-digit numbering system to identify their mutants. In the course of working with the mutants, we discovered that this system was extremely cumbersome and prone to typographical error. In addition, we have found that the literature describing the Dales mutants contains several errors attributable to this numbering system. We therefore adopted a revised numbering system, which we propose to be used universally in referring to vaccinia mutants (Tables 2-4, New system). The nomenclature uses the first letter of the last name for whom the collection is known, i.e., C for Condit, D for Dales, and E for Ensinger. The mutants are then designated as temperature-sensitive (ts) and are listed sequentially (i.e., Dales mutants are (Dts)1–97) (Tables 2–4). All 97 mutants Dales mutants have been assigned

TABLE 2

Mutants That Were Assigned to Complementation Groups

New system	Dales plaque isolate no.	ATCC number	Purified[a]	Dales EM category[b]	Map location[c]
Dts2	155	VR-3110	P	K	U4
Dts4	260	VR-3130	P	n.d.	U5
Dts8	991	VR-3121	NP	L	U5
Dts9	1085	VR-3131	P	E	D13
Dts10	1131	VR-3142	NP	C	A24*
Dts11	1327	VR-3102	NP	G,I	F10
Dts12	1408	VR-3112	NP	n.d.	D5
Dts14	1655	VR-3132	NP	B,P	A24*
Dts15	1911	VR-3143	NP	N	B1
Dts16	1923	VR-3103	NP	P	U6
Dts17	1988	VR-3113	P	n.d.	U6
Dts18	2047	VR-3123	NP	P	U7
Dts19	2096	VR-3133	P	B	E11
Dts20	2160	VR-3144	NP	P	U8
Dts22	3145	VR-3114	P	n.d.	G2
Dts23	3268	VR-3124	NP	n.d.	E(2–8)
Dts25	3498	VR-3145	NP	n.d.	E(2–8)
Dts27	3578	VR-3115	P	n.d.	D4
Dts28	3679	VR-3125	NP	G,P	A24*
Dts30	4149	VR-3146	P	DNA neg, B	D4
Dts33	5752	VR-3126	NP	P	U9
Dts35	5804	VR-3147	P	O	U5
Dts36	6003	VR-3107	NP	n.c.	U10
Dts38	6389	VR-3127	NP	DNA neg, C	D5
Dts40	6567	VR-3148	P	P	U11
Dts41	6606	VR-3108	NP	P	E(2–8)
Dts42	6626	VR-3118	NP	D	G2
Dts44	6741	VR-3138	NP	B	J4
Dts45	6754	VR-3149	NP	n.d.	A30, G(6–8)*
Dts46	6757	VR-3109	NP	F	A30, G(6–8)*
Dts47	6828	VR-3119	NP	P	U12
Dts48	6937	VR-3129	P	n.c.	U13
Dts49	7004	VR-3140	NP	G	A24*
Dts50	7014	VR-3139	P	n.d.	U10
Dts52	7160	VR-3160	NP	n.c.	A24*
Dts56	7473	VR-3151	NP	n.d.	D5
Dts57	7526	VR-3161	P	H	U14
Dts60	7672	VR-3189	P	n.d.	A24
Dts61	7726	VR-3152	NP	n.d.	U15
Dts62	7743	VR-3162	P	E	D13
Dts66	7954	VR-3153	P	n.c.	A24
Dts67	8125	VR-3163	NP	H,Q	I8
Dts68	8490	VR-3173	P	P	G(6–8)
Dts71	8643	VR-3154	NP	n.c.	U16
Dts77	8933	VR-3165	P	DNA neg, A, K	U17
Dts78	8946	VR-3175	NP	H,Q	U18
Dts80	9021	VR-3193	NP	H,Q	E(2–8)
Dts82	9139	VR-3166	NP	B	U19
Dts83	9153	VR-3176	P	J	U20
Dts85	9174	VR-3194	NP	C	J6
Dts86	9179	VR-3157	NP	n.c.	A24*
Dts88	9203	VR-3177	NP	E	D13
Dts89	9231	VR-3186	NP	C	G(6–8)
Dts90	9235	VR-3195	P	O	A24*
Dts93	9281	VR-3178	P	n.c.	U5
Dts94	9283	VR-3187	P	n.d.	A24*
Dts95	9312	VR-3196	P	n.d.	U21
Dts96	9383	VR-3159	NP	E,Q	D12
Dts97	9609	VR-3169	NP	B	U8

[a] P/NP = plaque purified/not plaque purified.
[b] n.c. = no category assigned by Dales. n.d. = no description available.
[c] For mapping nomenclature, see footnote a, Table 6.
* Unusual complementation, see text.

TABLE 3

Mutants Eliminated During Complementation Analyses

New system	Dales plaque isolate no.	ATCC number	Dales category[a]
Dts5	440	VR-3141	n.d.
Dts6	537	VR-3101	n.d.
Dts7	657	VR-3111	P
Dts13	1568	VR-3122	P
Dts24	3315	VR-3134	n.d.
Dts29	3681	VR-3135	I
Dts39	6501	VR-3137	H
Dts43	6712	VR-3128	G,P
Dts51	7133	VR-3150	M
Dts53	7221	VR-3170	I,P
Dts54	7316	VR-3179	Q
Dts55	7396	VR-3188	H
Dts69	8603	VR-3182	P
Dts70	8639	VR-3191	Q
Dts72	8697	VR-3164	G,P
Dts73	8741	VR-3174	DNA neg, B
Dts74	8743	VR-3183	H
Dts79	8987	VR-3184	n.c.
Dts81	9038	VR-3156	F
Dts87	9189	VR-3167	B
Dts91	9251	VR-3158	E,Q
Dts92	9276	VR-3168	P

[a] n.c. = no category assigned by Dales. n.d. = no description available.

ATCC numbers (Tables 2–4, ATCC number). The 59 mutants which were successfully characterized by complementation, plus Dts91 (see below), are being accessioned by ATCC for distribution and can be obtained once the accessioning is complete. The remaining 37

TABLE 4

Mutants Not Tested in Complementation Analyses Because of High Leakiness and High RI

New system	Dales plaque isolate no.	ATCC number	Dales category[a]
Dts1	27	VR-3100	M
Dts3	203	VR-3120	n.c.
Dts21	3060	VR-3104	n.d.
Dts26	3564	VR-3105	n.d.
Dts31	4213	VR-3106	n.d.
Dts32	5730	VR-3116	n.d.
Dts34	5796	VR-3136	n.c.
Dts37	6007	VR-3117	n.d.
Dts58	7565	VR-3171	H
Dts59	7634	VR-3180	n.d.
Dts63	7790	VR-3172	DNA neg, A
Dts64	7800	VR-3181	n.d.
Dts65	7883	VR-3190	H
Dts75	8788	VR-3192	n.c.
Dts76	8880	VR-3155	n.d.
Dts84	9170	VR-3185	J

[a] n.c. = no category assigned by Dales. n.d. = no description available.

TABLE 5

Mutants Reported by Dales But Not Deposited With the ATCC

Dales plaque isolate no.	Dales category[a]
146	n.c.
223	K
293	n.c.
1918	n.c.
2100	n.c.
2926	O
3216	n.c.
4094	H
5879	Q
7126	Q
7343	P
8845	B
9141	G,P
9194	DNAneg,C
9215	B
9223	B
9279	C
9330	C
9391	A
9433	B
9578	B

[a] n.c. = no category assigned by Dales.

Dales mutants will be archived by ATCC. In addition to simplifying the nomenclature, the new system has the advantage of resetting the references for Dales mutants to the isolates described here and accessioned by the ATCC, thus eliminating any further discrepancies in the literature.

There are some discrepancies between the collection as described by Dales *et al.* (1978) and the collection that we obtained from the ATCC. These discrepancies are illustrated in Tables 2–5. Specifically, Dales *et al.* published the isolation of 93 viruses, of which 79 were characterized by electron microscopy (Tables 1–6). We received 97 viruses that were deposited by Dales with the ATCC (Tables 2–4, Dts1–Dts97). Of the 93 viruses described by Dales, 23 were missing from the ATCC collection (Table 5). Twenty-seven of the 97 viruses that we received as the ATCC collection were not previously described in any form (Tables 2–4, n.d.) and 16 viruses were published but not categorized by morphology (Tables 2–5, n.c.). We have characterized the Dales mutants we received from the ATCC and noted the discrepancies.

All 97 mutants representing the ATCC (Dales) collection were grown in BSC40 cells at the permissive temperature, 31°C, and assayed for temperature sensitivity by plaque titration at 31 and 39.5°C. Each mutant was evaluated for potential fitness in the complementation assay using two measurements, the reversion index (RI) and the leakiness titer. The RI of each mutant was calculated by the equation: number of *large* plaques at 39.5°C/number of plaques at 31°C. The leakiness titer is defined as the number of *small* plaques at 39.5°C. The threshold values for keeping each mutant for complementation analysis required a RI of 10^{-4}-10^{-5} or lower and a leakiness titer of 10^4 or lower. These cutoffs are based on previous observations that mutants with high reversion indices may be difficult to grow without a substantial accumulation of revertants, and mutants with high values for leakiness or reversion may yield false positives in the complementation assay (Condit *et al.,* 1983). For those mutants with a RI on the borderline of our cutoff, several plaques were isolated and assayed for temperature sensitivity. In most cases this resulted in isolation of virus with a low enough RI for further analysis. These mutants are represented by a P under the column heading "Purified" in Table 2. In an effort to retain additional mutants that could theoretically represent unique mutations, we tested 10 mutants originally deemed too leaky for analysis (leakiness titer of 10^5 or higher at 39.5°C). We were unable to draw conclusions from 8 of the 10 mutants tested due to high backgrounds in the complementation analysis. We therefore did not pursue further analysis of mutants that we originally deemed too leaky. Of the original 97 viruses, 16 were deemed too leaky for use in the complementation assay (Table 4).

The assignment of each mutant to a complementation group was based on the qualitative spot test described previously by Condit and Motyczka (1981). Since we have access to the Condit collection, we were in possession of 32 defined complementation groups of vaccinia virus. We used at least one representative from each of the 32 Condit groups (Table 6, underlined) for complementation with the Dales mutants, thus avoiding the tedious work of performing pairwise infections for all of the Condit mutants. The remaining 81 mutants from the ATCC (Dales) collection, following analysis of RI and leakiness, were divided into five sets and tested for complementation. For each of the five sets, at a minimum, complementation was tested in pairwise infections with all of the mutants within the set, at least one representative from each of Condit's 32 groups, and a representative from each complementation group derived from each of the other four sets of Dales mutants. As final confirmation that all mutants in a given complementation group were in fact noncomplementing, pairwise spot tests were performed with all mutants in each group using a complementing mutant as a positive control. Examples of complementation tests performed for the purpose of confirming groups D5, G2, and U5 are shown in Fig. 1. Group D5 contains mutants Cts24, Dts12, Dts38, and Dts56 (rows A and B, Figs. 1a and 1c). Cts15, a mutant in the *F10L* gene, was the positive control for complementation in this group. Group G2 comprises the mutants Cts56, Dts22, and Dts42 (rows C and D, Figs. 1a and 1c). Cts15, an *F10L* mutant, and Cts33, a *D13L* mutant, were the positive controls. The unmapped complementation group U5 con-

TABLE 6

Complementation Analysis and Physical Mapping of Vaccinia Mutants

Map location[a]	Mutants[b]	Phenotype/function	Dales category[c]	References[d]
A1	Cts63	Defective late/VLTF		Carpenter and DeLange, 1992
A3	Cts8, Cts26	Normal/p4b core protein		S. Kato, N. Moussatché, R. C. Condit, unpublished data
A(8–17)	Cts40	Normal		
A18	Cts4, Cts22, Cts23	Abortive late/ATPase/DNA helicase/transcript release factor		Lackner and Condit, 2000
A24	Cts27, Cts29, Cts32, Cts47, Cts62, **Dts60(7672), Dts66(7954), Dts86(9179), Dts90(9235), Dts10(1131), Dts14(1655), Dts28(3679), Dts49(7004), Dts52(7160), Dts94(9283)**	Defective late/RNAP (132 kDa)/ IBT resistance	n.d., n.c., n.c., O, C, (B,P), (G,P), G, n.c., n.d.	Hooda-Dhingra *et al.,* 1990
A(25–29)	Cts6, Cts9	Normal		
A30	**Dts45(6754)?, Dts46(6757)?**		n.d., F	Szajner *et al.,* 2001
B1	Cts2*, *Cts3,* Cts25*, **Dts15(1911)**	DNA neg./protein kinase	N	Rempel and Traktman, 1992; Kovacs *et al.,* 2001
D2	Ets52, Ets94	Normal/virion core		Dyster and Niles, 1991
D3	Cts5, Cts35	Normal/virion core		Dyster and Niles, 1991)
D4	**Dts27(3578), Dts30(4149)***	DNA neg./uracil DNA glycosylase	n.d., B	Millns *et al.,* 1994
D5	Cts17*, Cts24*, Ets69, **Dts12(1408), Dts38(6389)*, Dts56(7473)**	DNA neg.	n.d., C, n.d.	McFadden and Dales, 1980; Evans and Traktman, 1992
D6	Cts46, Ets93	Normal/VETF subunit		Li *et al.,* 1994
D7	Cts21, Ets45	Defective late/RNAP (18 kDa)		Seto *et al.,* 1987
D11	Cts36, Cts50, Ets17	Defective late/ATPase/NPH-I		Christen *et al.,* 1998
D12	**Dts96(9383)**	VTF and capping enzyme subunit/VITF	(E,Q)	Carpenter and DeLange, 1991
D13	Cts33, Cts43, Ets101, **Dts9(1085), Dts62(7743), Dts88(9203)**	Normal/rifampicin resistance/ virion structure	E, E, E	Lake *et al.,* 1979; Seto *et al.,* 1987
E(2–8)a	Cts52, **Dts41(6606), Dts80(9021)**	Normal	P, (H,Q)	
E(2–8)b	Cts19, **Dts23(3268), Dts25(3498)**	Normal	n.d., n.d.	
E9	Cts42*, NGts26[e]	DNA neg./DNA polymerase/ PAA resistance		Traktman *et al.,* 1989
E11	Cts49, **Dts19(2096)**	Normal/core protein	B	Wang and Shuman, 1996
F10	*Cts12,* Cts15, Cts28, Cts54, Cts61, **Dts11(1327)**	Normal/virion protein kinase	(G,I)	Traktman *et al.,* 1995; Wang and Shuman, 1995
F(11–17)	Cts30, Cts48	Normal		
G2	Cts56, G2A, G2B, G2C, G2AS1, G2AS6, G2AS9, **Dts22(3145), Dts42(6626)**	Defective late/IBT dependent/ PTEF	n.d., D	Black and Condit, 1996; Condit *et al.,* 1996
G3	Cts60	Normal		Meis and Condit, 1991
G(6–8)	Cts11, Cts41, **Dts45(6754)?, Dts46(6757)?, Dts68(8490), Dts89(9231)**	Normal	n.d., F, P, C	Meis and Condit, 1991
H4	Cts1, Cts31, Cts55, Cts58	Normal/rap94		Kane and Shuman, 1992
I7	Cts16, Cts34	Normal/virion core		Ericsson *et al.,* 1995
I8	Cts10, Cts18, Cts38, Cts39, *Cts44,* **Dts67(8125)**	Normal/RNA helicase/NPH-II	(H,Q)	Gross and Shuman, 1996
J1	Cts45	Normal		R. C. Condit, unpublished
J4	Cts7, Cts20, **Dts44(6741)**	Defective late/RNAP (22 kDa)	B	Hooda-Dhingra *et al.,* 1989
J6	Cts51, Cts53, Cts65, **Dts85(9174)**	Defective late/RNAP (147 kDa)	C	Hooda-Dhingra *et al.,* 1989
U1	Cts57	Normal		
U2	Cts37	Normal		
U3	Cts13, Cts64	Normal		
U4	**Dts2(0155)**		K	
U5	**Dts4(0260), Dts8(0991), Dts35(5804), Dts93(9281)**		n.d., L, O, n.c.	
U6	**Dts16(1923), Dts17(1988)**		P, n.d.	
U7	**Dts18(2047)**		P	
U8	**Dts20(2160), Dts97(9609)**		P, B	
U9	**Dts33(5752)**		P	
U10	**Dts36(6003), Dts50(7014)**		n.c., n.d.	

TABLE 6—*Continued*

Map location[a]	Mutants[b]	Phenotype/function	Dales category[c]	References[d]
U11	**Dts40(6567)**		P	
U12	**Dts47(6828)**		P	
U13	**Dts48(6937)**		n.d.	
U14	**Dts57(7526)**		H	
U15	**Dts61(7726)**		n.d.	
U16	**Dts71(8643)**		n.c.	
U17	**Dts77(8933)**		(A, K)	McFadden and Dales, 1980; Millns *et al.,* 1994
U18	**Dts78(8946)**		(H, Q)	
U19	**Dts82(9139)**		B	
U20	**Dts83(9153)**		J	
U21	**Dts95(9312)**		n.d.	

[a] A specific letter and number combination indicates that the mutants in this complementation group have been mapped to a specific gene. A letter and range of numbers in parentheses indicates that the mutants are in the same complementation group but are not mapped to a single open reading frame; an approximate range is indicated, using gene numbers. The letter U indicates that the mutants are not mapped and the number is arbitrary to help distinguish the unmapped groups from one another.

[b] Mutants are listed by the new nomenclature (Dtsxx or Ctsxx) with the four-digit number identification used by Dales shown in parentheses. Mutants that are underlined were used as a representative of that group in complementation. The mutants shown in bold are new to the complementation group and are described in this article. Mutants shown in italics indicate viruses that may contain more than one mutation.

[c] The category for each Dales mutant is listed and corresponds to the order in which they are listed at the left.

[d] Initial characterization of all Condit mutants is described in Condit and Motyczka (1981), Condit *et al.* (1983), and Thompson and Condit (1986). For Condit mutants that have been characterized further, the most recent additional references are provided. Initial characterization of all Dales mutants described in Dales *et al.* (1978). For Dales mutants that have been characterized further, a prototypical reference is provided. See text for additional references.

[e] Described in Sridhar and Condit (1983).

***** DNA negative mutant.

[?] Unusual complementation, see text.

n.c. = no category assigned by Dales. n.d. = no description available.

sists of mutants Dts4, Dts8, Dts35, and Dts93 (Figs. 1b and 1d). Cts63, an *A1L* mutant, and Cts15, an *F10L* mutant, were the positive controls for complementation. In all cases, the complementation assays demonstrate the absence of plaques in the presence of two noncomplementing mutants (for example, Cts24 and Dts12, Figs. 1a and 1c, row A, well 1) and the formation of plaques when two mutants complement one another (for example, Cts24 and Cts15, Figs. 1a and 1c, row A, well 4). The single infection with each mutant was negative, i.e., no macroscopic plaques were detected (data not shown).

During complementation analysis 22 mutants complemented poorly or in an anomalous manner and were dropped from consideration (Table 3). Table 3 also includes the eight mutants mentioned above that were originally deemed too leaky but for which complementation was nevertheless attempted and which subsequently were dropped from further consideration for anomalies observed during complementation. The final results of the complementation assays are shown in Table 6 with the new mutants in each group shown in bold. The final compilation contains 132 mutants from three collections, together representing 53 complementation groups, 32 of which have been mapped or partially mapped to vaccinia genes and 21 of which are unmapped. Each of the complementation groups containing new mutants is discussed further below.

A24: The 132-kDa RNA polymerase subunit

The members of the A24 group as defined by complementation and/or marker rescue include five mutants from the Condit collection (Cts27, Cts29, Cts32, Cts47, Cts62) and 10 mutants from the Dales collection (Dts10, Dts14, Dts28, Dts49, Dts52, Dts60, Dts66, Dts86, Dts90, Dts94) (Table 6). The Condit mutants were previously mapped by marker rescue to the *A24R* gene, the 132-kDa vaccinia RNA polymerase subunit (RNAP) (Thompson and Condit, 1986; Patel and Pickup, 1989; Hooda-Dhingra *et al.,* 1990). The A24 group demonstrated some unusual complementation properties. Previous complementation analysis with the five Condit mutants revealed that Cts27, Cts29, Cts32, and Cts47 were noncomplementing in all pairwise crosses. Cts62 was noncomplementing with only Cts47 while complementing Cts27, Cts29, and Cts32. It was concluded that Cts62 underwent intragenic complementation with the three mutants Cts27, Cts29, and Cts32 (Hooda-Dhingra *et al.,* 1990). In this study, all of the Dales mutants were noncomplementing with each

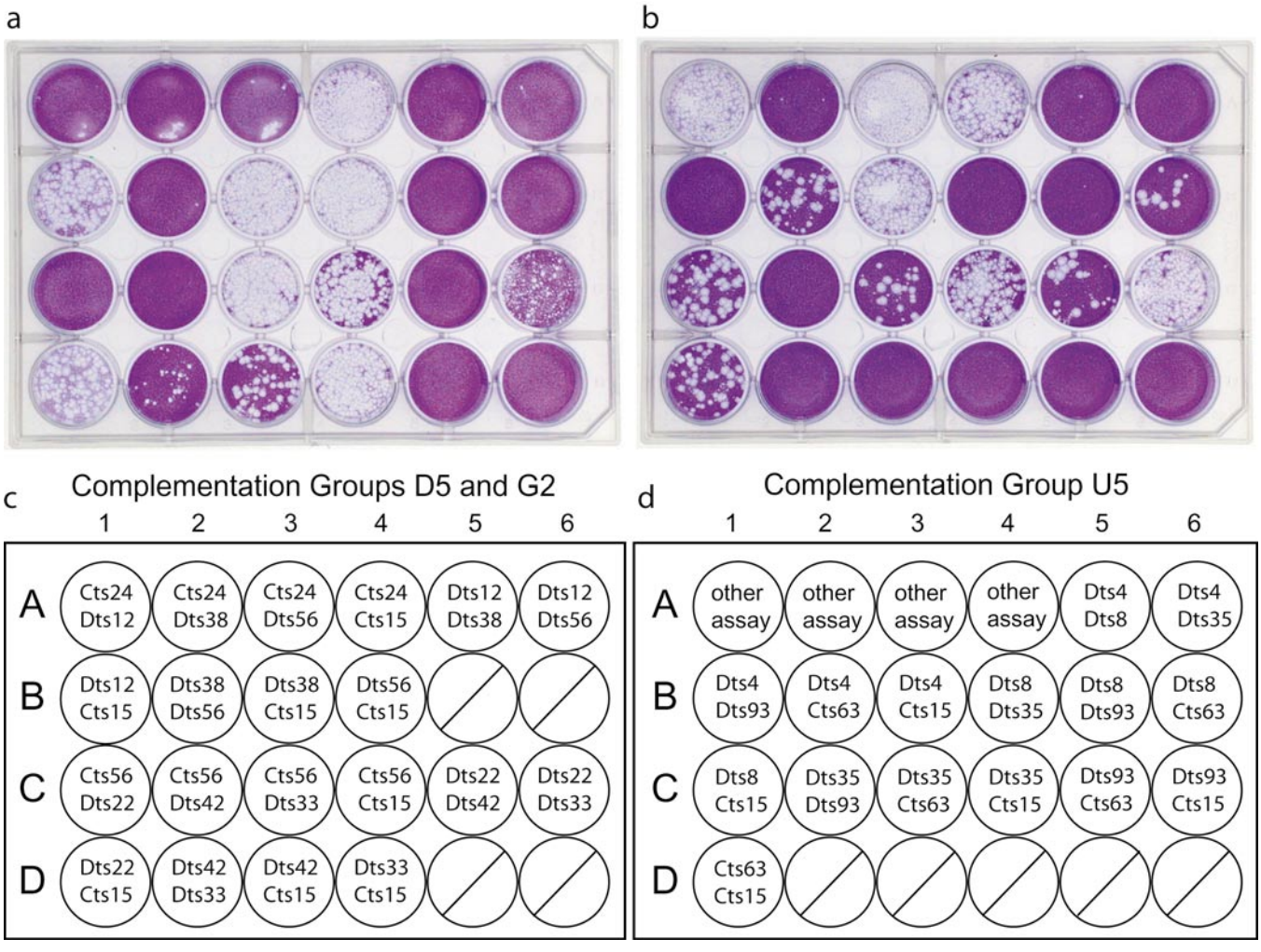

FIG. 1. Qualitative complementation tests. (a) A crystal violet-stained 24-well dish showing results of complementation within Groups D5 and G2. Complementing mutants Cts15 and Dts33 were included as positive controls. The presence of plaques in a given well indicates that the two mutants are complementing. The absence of plaques in a well indicates that the two mutants are noncomplementing and are thus in the same complementation group. (b) Complementation analysis of mutants in the unmapped group U5 including Cts15 and Cts63 as positive controls. (c and d) A diagram showing, by mutant, which viruses were added to each well on the plates in (a) and (b), respectively. The wells designated with "other assay" contain viruses used in a complementation assay not described here. The wells containing a slash did not contain any virus.

other, a result confirmed in three separate complementation experiments. Complementation analysis with the Dales and Condit mutants was slightly more complex. Three Condit mutants (Cts27, Cts29, Cts47) were used as representatives and were noncomplementing with one another and with two Dales mutants, Dts60 and Dts66. Two other Dales mutants showed selective noncomplementation with the Condit mutants. Dts86 was noncomplementing with both Cts29 and Cts47 but complemented Cts27. Dts90, on the other hand, was noncomplementing only with Cts47. These results are not inconsistent with the results reported for complementation with the five Condit mutants. The Dales mutants that complement the Condit mutants may represent additional cases of intragenic complementation.

We performed marker rescue with all 13 mutants in an attempt to clarify these complementation discrepancies (data not shown). Two DNA clones were used for marker rescue, pWR 112–149 and pTA-A24. The cosmid pWR 112–149 was one of three cosmid clones previously used to map Cts27, Cts29, and Cts47 to the *Hin*dIII A fragment (Hooda-Dhingra *et al.,* 1990). pTA-A24 contains the precise open reading frame of *A24R*. Cts27, Cts29, Dts60, and Dts66 demonstrated rescue with both pWR 112–149 and pTA-A24 as compared to a no DNA control. Dts90 and Dts94 were also rescued by pWR 112–149 and pTA-A24; however, both mutants formed small plaques in the presence of either DNA clone. This may suggest the presence of a second mutation outside of *A24R* in these mutants. The remaining mutants, Cts47, Dts10, Dts14, Dts28, Dts49, Dts52, and Dts86, were too leaky to be assessed accurately in the tests performed here. Our marker rescue results unambiguously support the assignment of at least two of the Dales mutants to A24. In

addition, results reported here, plus previous results, support the idea that mutants in this group can display intragenic complementation.

Previous studies on the five Condit mutants comprising the A24 group revealed a phenotype described as defective late (described in more detail under Introduction). This phenotype was heterogeneous as exemplified by the Condit A24 mutants. Cts29 and Cts47 were strongly defective late mutants. Neither mutant synthesized late proteins at 40°C and both were impaired in host and early protein synthesis shutoff. Cts27 and Cts32 demonstrated a weaker phenotype. The synthesis of late proteins was delayed in Cts27 and Cts32 but the delay was equivalent at both 31°C and 40°C, as was the amount of protein synthesis. Cts62 defined yet another phenotype in this group with normal quantities of late protein synthesis beginning at the same time at both 31 and 40°C, whereas in a wt infection, late protein synthesis generally begins earlier at 40°C than at 31°C. The growth phenotype and complementation of the Condit mutants suggested that the mutations affected biochemically different functions of A24. First, the growth phenotype did not correlate with the results of protein synthesis analysis. Both Cts27 and Cts32 grew normally at 31°C but exhibited an impaired pattern of viral protein synthesis. Cts62, on the other hand, did not grow at 40°C yet exhibited a normal pattern of protein synthesis. The heterogeneity in phenotype was accounted for by the hypothesis that in strongly defective late mutants the mutation destroys a larger percentage of the RNA polymerase activity than in weakly defective late mutants (Hooda-Dhingra *et al.,* 1990). This heterogeneity of phenotype is consistent with the heterogeneous EM phenotype of the 10 Dales mutants assigned to this group (Table 6). The Dales mutants assigned to the *A24R* gene include diverse morphological phenotypes ranging from categories B to Q (Tables 1 and 6). As demonstrated by other Dales mutants (Lake *et al.,* 1979; McFadden and Dales, 1980), mutants with similar morphological phenotypes are not necessarily closely related genetically.

The only Dales mutant in this group for which a protein synthesis phenotype has been described, Dts86, expressed both early and late functions very slowly, consistent with the assignment of this mutant, and therefore the rest of the complementation group, to the A24 group (Wilton and Dales, 1989). Dts86 was also analyzed for its affect on migration of cellular RNA polymerase II (RNAPII). Silver *et al.* (1979) had reported that host RNAPII migrated to the cytoplasm during a vaccinia infection and that in a Dts86 infection this transfer did not occur (Wilton and Dales, 1989). It was concluded that the migration of RNAPII may be influenced by overall rates of vaccinia virus specific synthesis and that absence of RNAPII in the cytoplasm of a Dts86 infection was a result of delayed early synthesis.

An additional mutant from the Dales collection, Dts91, was dropped during complementation for an even more complicated complementation phenotype (Table 3). Dts91 was noncomplementing with Dts90 but complemented the other nine Dales mutants in this group. The mutant was not tested against the Condit mutants. Dts91 was particularly interesting since it was the only mutant from 11 tested mutants that had a restriction fragment length polymorphism associated with the ts locus (McFadden *et al.,* 1980). This mutant acquired a novel *Eco*RI-restriction site in a restriction fragment corresponding to the *Eco*RI fragment D of rabbitpox virus, the only *Eco*RI restriction map available at the time. The rabbitpox D fragment had been shown to reside within the *Hin*dIII A fragment (McFadden *et al.,* 1980). Assuming that rabbitpox and IHD-W are similar in *Eco*RI fragment D (Schumperli *et al.,* 1980), these results are not inconsistent with placement of Dts91, as well as the other mutants of this complementation group, in *A24R*.

Two-dimensional gel analysis of extracts from cells infected with Dts91 revealed a 37-kDa protein with an altered charge as compared to extracts from wt infected cells (McFadden *et al.,* 1980). Four independent ts^+ revertants of Dts91 were isolated. Three of the four revertants lost the additional *Eco*RI site found in the mutant virus and had a 37-kDa protein mobility identical to wt IHD-W. The fourth revertant retained the mutant restriction pattern but had a 37-kDa protein mobility intermediate between wt and Dts91. McFadden *et al.* (1980) concluded that this fourth revertant was an intragenic suppressor containing a second mutation within the same gene at a different locus. Obviously the 37-kDa protein is too small to be the 132-kDa RNAP subunit (encoded by gene *A24R*); however, the 35-kDa RNAP subunit is close in location on the genome (gene *A29L*). Thus one hypothesis which could explain the charge alteration to the 37-kDa protein in Dts91 is that Dts91 contains a mutation in *A29L*. This raises several possibilities concerning the location of the Dts91 mutation. First, the Dts91 mutation may be in the *A29R* gene and its noncomplementation with Dts90 may indicate that Dts90 is a double mutant, containing both *A24R* and *A29L* mutations. Second, the Dts91 mutant may have more than one mutation, one in the *A24R* gene and a second in the *A29L* gene. Complementation of this mutant may depend on the location of the *A24R* mutation in the second mutant virus. A third possibility is that the Dts91 mutation affects an activity of the A24 protein that has previously remained uncharacterized. This may involve a protein modification activity such as a kinase or a glycosylase, thus affecting the mobility of the 37-kDa protein in an indirect manner. Dts91 was dropped from our final results due to the complicated complementation phenotype. For this same reason, Dts91 is an interesting mutant that poses intriguing questions.

Dts91 was categorized as a group E mutant in the Dales EM phenotype scheme (Table 1) (Dales *et al.,*

1978). The other group E mutant viruses contain mutations in either gene *D12L* or *D13L*. The group E mutants, in general, demonstrated a defect in membrane self-assembly and maturation. Dts91, however, behaved anomalously at 40°C. The morphogenesis of this mutant was termed "leaky" as both abnormal envelope formation and the development of mature virions were observed. However, Dts91 was "tight" in terms of infectivity (as measured by plaque assay) (Lake *et al.,* 1979). Additionally, this was the only group E mutant with no defect in the processing of precursor polypeptides (McFadden *et al.,* 1980). In summary, Dts91 was different from the other described group E mutants and, in fact, had a leaky morphology phenotype that may also affect complementation of this mutant.

The simplest explanation for our results is that all of the mutants (Dts10, Dts14, Dts28, Dts49, Dts52, Dts60, Dts66, Dts86, Dts90, and Dts94) contain at least one mutation in *A24R*. We therefore chose to assign these mutants to the A24 complementation group with the caveat that further use of these mutants requires that the mutations be accurately mapped to the *A24R* gene.

B1: A serine/threonine protein kinase

Complementation analysis placed one additional mutant from the Dales collection (Dts15) in the B1 group that consisted of three mutants from the Condit collection (Cts2, Cts3, Cts25) (Table 6). B1 is a 300 amino acid protein that is expressed early during infection. It encodes a protein kinase with specificity for serine and threonine residues and is known to phosphorylate a vaccinia late transcription factor encoded by the *H5R* gene and the ribosomal proteins, Sa and S2 (Rempel and Traktman, 1992; DeMasi and Traktman, 2000). *B1R* is also one of four genes known to have DNA negative ts mutant phenotypes.

Initial characterization showed that the Condit mutants Cts2 and Cts25 were DNA negative as evidenced by the absence of incorporation of [^{3}H]thymidine into acid-precipitable material. Interestingly, Cts3 was not DNA negative in this assay (Condit and Motyczka, 1981; Condit *et al.,* 1983). Further analysis of Cts3 was not performed because it could not be mapped by marker rescue and was thought to contain two mutations (Rempel *et al.,* 1990). Subsequently, Rempel *et al.* (1990) analyzed both Cts2 and Cts25 for DNA replication in mouse L cells and BSC40 cells. It was found that both mutants displayed a host range where the restriction on DNA replication was 10-fold more severe and the restriction on viral yield was 20- to 135-fold more complete in L cells than BSC40 cells. The two mutants exhibited different plaque phenotypes on BSC40 cells. Cts25 produced no macroscopic plaques where Cts2 produced minute plaques on BSC40 cells at 39.5°C. Both mutants produced unstable B1 proteins; however, the Cts2 B1 protein was unstable at both temperatures, whereas the Cts25 B1 protein was specifically unstable at the nonpermissive temperature. Kovacs *et al.* (2001) found that Cts25 also had a ts defect in intermediate gene expression independent of the effects on viral DNA replication. This is consistent with the fact that Cts25 directed synthesis of up to 60% of wt DNA levels but only 15% of viable progeny and no plaques on BSC40 cells (Kovacs *et al.,* 2001).

Dts15 is the only mutant categorized as group N of the Dales morphological aberrations (Table 1) (Dales *et al.,* 1978). Using electron microscopy, this mutant was shown to produce factories that contained immature and numerous intermediate maturing forms of virus. This mutant was classified by Dales as DNA positive using [^{3}H]thymidine pulse labeling. This method is considered to be the most stringent since it only measures DNA synthesis activity during isolated periods (<5 h into replication) of infection (Rempel *et al.,* 1990). It is perhaps possible that the DNA negativity of this mutant was missed due to the stringent assay used for characterization. However, the DNA negative mutants defined by Dales were all characterized in groups A–D and the grouping of this mutant in group N seems to argue that this mutant is not DNA negative. In summary, the phenotypes of mutants in the B1 group are heterogeneous, even with respect to DNA replication. This may reflect a multifunctional role for the B1 kinase in virus infection. Nevertheless, attempts should be made to map Dts15 to the *B1R* open reading frame prior to further analysis.

D4: Uracil DNA glycosylase

The members of the D4 complementation group include two mutants from the Dales collection (Dts27, Dts30) (Table 6). The vaccinia virus uracil DNA glycosylase gene *D4R* was originally identified based on homology to the Shope fibroma virus (SFV) uracil DNA glycosylase gene (Stuart *et al.,* 1993). Identification of a temperature-sensitive mutant in *D4R* was independently revealed when marker rescue mapped the mutation in Dts30 to the *D4R* gene (Millns *et al.,* 1994). A uracil DNA glycosylase acts at the initial stage of the pathway for excision repair by catalyzing the hydrolysis of uracil residues that have been introduced into DNA either through misincorporation of dUTP or through deamination of cytosine. This activity was rapidly induced following a poxvirus infection, suggesting that the enzyme was required prior to and during DNA synthesis (Stuart *et al.,* 1993).

Dts30 was characterized by Dales to reside within the B group of morphological aberrations (Table 1) (Dales *et al.,* 1978). At the permissive temperature, Dts30 DNA replication reaches its peak synthesis several hours later than wt virus and synthesizes less than 50% of the wt levels of DNA (McFadden and Dales, 1980; Millns *et al.,* 1994). At the nonpermissive temperature, Dts30 pro-

duces very little or an undetectable quantity of DNA and continues to synthesize the early proteins, classifying the mutant as DNA negative (Dales *et al.,* 1978; McFadden and Dales, 1980; Millns *et al.,* 1994). The Dts30 ts mutation, a glycine to arginine substitution, was found to reside at amino acid 179 close to the active site, histidine 181, of the enzyme (Millns *et al.,* 1994; Ellison *et al.,* 1996). Our complementation results placed Dts27 within the same complementation group as Dts30. There is no published description of this mutant from which to speculate on its phenotype relative to Dts30.

D5: An ATPase required for DNA replication

The members of the D5 group include two mutants from the Condit collection (Cts17, Cts24), one mutant from the Ensinger collection (Ets69), and three mutants from the Dales collection (Dts12, Dts38, Dts56) (Table 6). The D5 protein is an ATPase required for DNA replication *in vivo* (Evans *et al.,* 1995). In our experiments Cts24 was used as the Condit collection representative and was found to be noncomplementing with Dts12, Dts38, and Dts56 (Figs. 1a and 1c, rows A and B). Evans and Traktman (1992) independently confirmed that Cts17 was noncomplementing with Dts38. Four of these mutants were characterized as DNA negative, Cts17, Cts24 (Condit and Motyczka, 1981), Ets69 (Seto *et al.,* 1987), and Dts38 (Dales *et al.,* 1978; McFadden and Dales, 1980). The two remaining mutants, Dts12 and Dts56, have not been previously characterized.

The precise role of D5 during infection has not been established. A shift in temperature from permissive to nonpermissive in Cts17, Cts24, and Dts38 infections resulted in an abrupt cessation of DNA synthesis also known as a "fast-stop" phenotype. This provided evidence that these mutants were defective in chain elongation and that the mutation affected a gene product intimately associated with the replication fork (McFadden and Dales, 1980; Evans and Traktman, 1992). D5 has no homology to known replication proteins or helicases, but has a motif associated with ATP and GTP binding found in many nucleotide hydrolases involved in DNA replication. Purified D5 protein demonstrated ATPase activity that was not stimulated by any common nucleic acid cofactor and used all eight ribo- and deoxyribonucleoside triphosphates tested as substrates with the greatest activity resulting from CTP and TTP (Evans *et al.,* 1995). Interestingly Cts17, Cts24, and Dts38 all contained N-terminal lesions in *D5R* and possessed an unexplained defect in homologous recombination and marker rescue, suggesting that D5 may play a role in both DNA replication and recombination (Evans and Traktman, 1992). The combination of the fast-stop phenotype and the link to DNA recombination supports a role for D5 in the unwinding and/or annealing of DNA strands (Evans *et al.,* 1995).

D12: The small subunit of the mRNA capping enzyme and VTF

Dts96 was previously mapped to *D12L* by Carpenter and DeLange (1991) (Table 6). *D12L* encodes both the small subunit of the mRNA capping enzyme and the early transcription termination factor, VTF. Dts96 is a group E mutant characterized by morphology similar to the effects of rifampicin on a wt infection (Table 1) (Dales *et al.,* 1978; Lake *et al.,* 1979). At both permissive and nonpermissive temperatures, there was no delay in DNA replication or protein synthesis (Carpenter and DeLange, 1991). However, there was a temperature-dependent defect in telomere resolution. Protein analysis revealed that polypeptides p18.5 and p18 were missing or present in minute quantities, indicating a defect in posttranslational cleavage (Lake *et al.,* 1979). This mutant was found to have a single point mutation located at nucleotide 379 in gene *D12L* that is responsible for the temperature-sensitive and telomere resolution phenotypes (Carpenter and DeLange, 1991). Our complementation analysis did not reveal any additional mutants in *D12L*.

D13: The p65 scaffold protein

Members of the D13 group include two mutants from the Condit collection (Cts33, Cts43), one mutant from the Ensinger collection (Ets101), and three mutants from the Dales collection (Dts9, Dts62, Dts88) (Table 6). The *D13L* gene encodes a 65-kDa protein, designated p65, that is thought to function as a scaffold protein for the formation of viral crescents and immature virions (Sodeik *et al.,* 1994). The three Dales mutants, Dts9, Dts62, and Dts88, are members of the group E morphology group (Table 1) (Dales *et al.,* 1978). A previous report of complementation among the group E mutants assigned Dts9 and Dts88 to different complementation groups, contradictory to our data. However, in the same report Dts62 was found to be noncomplementing with both Dts9 and Dts88, supporting the assignment of the three mutants to the same complementation group (Lake *et al.,* 1979). In addition, Carpenter and DeLange (1991) reported that Dts9, Dts62, Dts88, and Cts33 are noncomplementing and their marker rescue analysis supported the assignment of these four ts mutants to *D13L*. Our results confirm that Dts9, Dts62, and Dts88 are all noncomplementing with one another as well as the Condit collection representative, Cts33, further supporting the assignment of these three mutants to the D13 complementation group.

The antibiotic rifampicin has been shown to inhibit the maturation of poxviruses at a discrete step in envelope formation (Moss *et al.,* 1969). In the presence of rifampicin, the membranes associated with the viral factories are irregularly shaped, and crescents and spherical, immature particles do not form (Grimley *et al.,* 1970). Mutant viruses that are resistant to rifampicin have been mapped to the *D13L* open reading frame (Tartaglia and

Paoletti, 1985; Baldick and Moss, 1987). The Dales group E mutants possess a morphological phenotype that mimics the defects seen in the presence of rifampicin. The mutant defects include the formation of numerous viroplasmic foci, each surrounded by flexible envelope segments and membrane structures that are devoid of "spicules," similar to the aberrant membranes formed in the presence of rifampicin (Dales *et al.,* 1978). Two groups have demonstrated using immunolocalization and electron microscopy that p65 is localized in viral factories, specifically to the concave surface of viral crescents (Vanslyke and Hruby, 1994; Sodeik *et al.,* 1994). In the presence of rifampicin, p65 is found in unique structures that are referred to as p65 bodies. The rifampicin bodies, present only in the presence of rifampicin, contain precursor membranes that are not labeled with p65 antibodies. After removal of the drug, the rifampicin block is reversed and p65 antibodies again label viral crescents. These results suggest that the formation of crescents requires the association of p65 with the membrane precursors. Thus, it has been proposed that p65 functions as an internal scaffold protein (Sodeik *et al.,* 1994).

E(2–8): Two complementation groups

Two mutants from the Condit collection map to the region between E2 and E8 but complement one another. These mutants, Cts52 and Cts19, therefore represent two complementation groups, E(2–8)a and E(2–8)b, that map to this region but whose exact location is not known (Condit and Motyczka, 1981; Condit *et al.,* 1983; Thompson and Condit, 1986). We have identified two new Dales mutants by complementation analysis to add to each of these groups. Those mutants that are noncomplementing with Cts52 and are thus members of group E(2–8)a include Dts41 and Dts80 (Table 6). Cts52 was reported to have a normal protein synthesis phenotype (Condit *et al.,* 1983). Dts41 was assigned to Dales category of morphological aberration group P, while Dts80 was assigned to both H and Q (Table 1). Both Dts41 and Dts80 therefore produced nearly normal or normal particles by electron microscopy, consistent with the Cts52 normal phenotype (Dales *et al.,* 1978). Mutants that did not complement Cts19 but were complementing with Cts52 include Dts23 and Dts25, thus forming group E(2–8)b (Table 6). Cts19 was also reported to have a normal phenotype (Condit and Motyczka, 1981). No description of Dts23 or Dts25 has been published.

E11: A core protein

One mutant from the Condit collection (Cts49) was mapped to the *E11* gene and is noncomplementing with one mutant from the Dales collection (Dts19) (Table 6). E11 is a 129 amino acid protein that is synthesized late during infection. The protein was found in deoxycholate extract of virus cores and the insoluble core fraction but not the envelope fraction, evidence that E11 is a component of the vaccinia core. The mutation in Cts49 has been mapped as a glycine-to-arginine substitution at amino acid 66 (Wang and Shuman, 1996). Cts49 has a normal phenotype with no defect in transcription or posttranslational cleavage (Condit *et al.,* 1983; Wang and Shuman, 1996). Electron microscopy of Cts49-infected cells showed normal appearing IMV particles at 40°C and a lack of aberrant viral structures at either 31 or 40°C (Wang and Shuman, 1996). This is in contrast to Dts19, which was assigned to category B of morphological aberrations (Table 1). Cells infected with Dts19 contained only foci of viroplasm and did not show development of any membrane structures (Dales *et al.,* 1978). As previously described for both groups A24 and B1, differences in the morphology of mutants that are noncomplementing do not preclude the assignment of the viruses to the same complementation group. Given the discrepancy in phenotype, however, caution should be taken prior to analysis of Dts19 to ensure that this mutant maps to *E11*.

F10: A serine/threonine kinase

The members of the F10 complementation group include five mutants from the Condit collection (Cts12, Cts15, Cts28, Cts54, Cts61) and one mutant from the Dales collection (Dts11) (Table 6). *F10L* encodes a serine/threonine kinase that represents the major kinase activity in the virion. The Condit mutants, Cts15, Cts28, Cts54, and Cts61, were mapped by marker rescue to the *F10L* gene. Cts12, on the other hand, could not be mapped by either Wang and Shuman (1995) or Traktman *et al.* (1995) and was thought to contain one or more additional mutations located outside of *F10L*. The four Condit mutants were found to have normal protein synthesis but varied in the severity of a block in p4a and p4b processing. Cts61 converts p4b to 4b but does not process p4a to 4a. No mature progeny virions were observed at 40°C by electron microscopy in Cts28, Cts54, and Cts61 infections. Neither Cts15 nor Cts28 demonstrated signs of virion morphogenesis or spherical immature particles, and in both mutants there was scant formation of viral membranes (Traktman *et al.,* 1995; Wang and Shuman, 1995). Reports on Cts54 differ, indicating either that these infections may contain only foci of viroplasm that are occasionally accompanied by viral membrane fragments (Wang and Shuman, 1995) or that these infections contain crescent and immature virions without nucleoids (Traktman *et al.,* 1995). Cts61 demonstrated approximately 50% of infected cells that contained sizable virosomes devoid of any virus maturation and 50% that contained crescents and immature virions without nucleoids (Traktman *et al.,* 1995; Wang and Shuman, 1995). These phenotypes are in agreement with observed leakiness in a plaque assay: Cts15 and Cts28 did not form macroscopic plaques at 39.5°C and Cts54

and Cts61 are far leakier, forming minute plaques at 39.5°C (Traktman *et al.,* 1995). Dts11 was categorized in Dales groups G and I where incomplete membranes with spicules and some complete immature particles were observed (Table 1) (Dales *et al.,* 1978). In our hands, Dts11 was also slightly leaky, scarcely making the cutoff for useful mutants. The Dts11 phenotype is consistent with the phenotype described for Cts54 and Cts61.

F10 is expressed late during infection and it is the major kinase activity in the virion (Wang and Shuman, 1995). A shift in temperature from 39.5 to 32°C in a Cts28 infection resulted in a large burst of Cts28 virus. When the same temperature shift was performed with the addition of rifampicin at the time of shift, no burst of virus was detected. This indicated that arrest in a Cts28 infection occurs prior to the rifampicin-sensitive step. The F10 mutants therefore define the earliest genetic defined step in vaccinia virus assembly (Traktman *et al.,* 1995; Wang and Shuman, 1995). The temperature-sensitive defect in the F10 mutants was proposed to be the result of failure to phosphorylate one or more viral proteins that are necessary for membrane formation. Several vaccinia proteins are phosphorylated *in vivo* and have been suggested to be the target of F10. These proteins include F17, H5, and A17 (Traktman *et al.,* 1995; Wang and Shuman, 1995; Betakova *et al.,* 1999).

G2: Positive transcription elongation factor

The G2 complementation group includes one original randomly isolated ts mutant from the Condit collection (Cts56) and two ts mutants from the Dales collection (Dts22, Dts42). Additional mutants were created that contain either frameshifting deletions in the *G2R* gene (G2A, G2B, G2C) or engineered ts lesions, resulting in the loss of G2 expression (G2AS1, G2AS6, G2AS9) (Table 6) (Meis and Condit, 1991; Hassett and Condit, 1994). *G2R* encodes a 26-kDa protein expressed early during infection that has been proposed to function as a postreplicative positive transcription elongation factor (Black and Condit, 1996). A complementation test of the G2 mutants is shown in Figs. 1a and 1c (rows C and D). Cts15 and Dts33 are used in this experiment as positive controls for complementation. Clearly, the absence of plaques in wells 1, 2, and 5 (Figs. 1a and 1c, row C) demonstrates the lack of complementation between Cts56, Dts22, and Dts42 as compared to the presence of plaques in row C wells 3, 4, 6 and row D, wells 1–4 (Figs. 1a and 1c).

Characterization of the G2 mutants Cts56 and G2A revealed a defective late phenotype (Condit *et al.,* 1983; Black and Condit, 1996). These viruses synthesize early viral mRNA, early viral proteins, and viral DNA normally under nonpermissive conditions. Intermediate and late viral RNA synthesis begins normally but these mRNAs are 3′ truncated and reduced in amount. This results in the synthesis of mRNAs that are too short to encode large late proteins. Base on these results, the G2 protein was proposed to function as a postreplicative positive transcription elongation factor, such that in the absence of G2 protein synthesized transcripts are shorter than in the presence of G2 protein.

The G2 mutants also demonstrate an altered phenotype in the presence of the anti-poxviral drug isatin-β-thiosemicarbazone (IBT). In our hands, the differences in response to IBT have indicated a variation in the activity of the *G2R* gene product (Hassett and Condit, 1994). Viruses that are wild-type for G2 activity are IBT-sensitive (Fig. 2, IHD-W) and viruses such as G2A, G2B, and G2C, that have no G2 function, are IBT-dependent (Meis and Condit, 1991). IBT resistance presumably reflects an intermediate level of G2 activity. The IBT phenotype relates to the G2 mutant phenotype. IBT treatment enhances transcription elongation (Bayliss and Condit, 1993; Xiang *et al.,* 1998), compensating for the elongation defect in G2 null mutants, hence, the IBT-dependent phenotype of G2 null mutants. The temperature-sensitive mutant Cts56 is IBT-resistant at 31°C, indicating that even at the permissive temperature this virus is somewhat compromised for G2 activity. The IBT dependence of Cts56 at 39.5°C reflects the absence of G2 activity at the nonpermissive temperature (Fig. 2, Cts56) (Meis and Condit, 1991).

Description of the phenotypes of the Dales G2 mutants includes only Dts42. This mutant was categorized in Dales group D, mutants that contain foci of DNA viroplasm and DNA paracrystals (Table 1) (Dales *et al.,* 1978). No published description is available for Dts22. Neither Dts22 nor Dts42 were characterized for transcriptional defects. As further evidence to support the assignment of Dts22 and Dts42 to the G2 complementation group, we performed a plaque assay in the presence and absence of IBT at both 31 and 39.5°C (Fig. 2, Dts22 and Dts42). Under these conditions the phenotype of both viruses resembled that of Cts56. Specifically, both Dts22 and Dts42 were IBT-resistant at 31°C and IBT-dependent at 39.5°C. Thus, the phenotypes of Dts22 and Dts42 with respect to IBT are consistent with the placement of these mutants in the G2 complementation group.

A30 and G(6–8): A complicated complementation

Our complementation results placed two Condit collection mutants (Cts11, Cts41) and four mutants from the Dales collection (Dts45, Dts46, Dts68, Dts89) in the same group (Table 6). All six of these mutants were noncomplementing in three separate complementation tests. This complementation result is paradoxical because Dts46 has been mapped to gene *A30* (Szajner *et al.,* 2001), whereas Cts11 and Cts41 have been mapped to the right half of the *Hin*dIII G fragment, comprising genes *G6* through *G8* (Meis and Condit, 1991). In an attempt to resolve this paradox, we performed marker

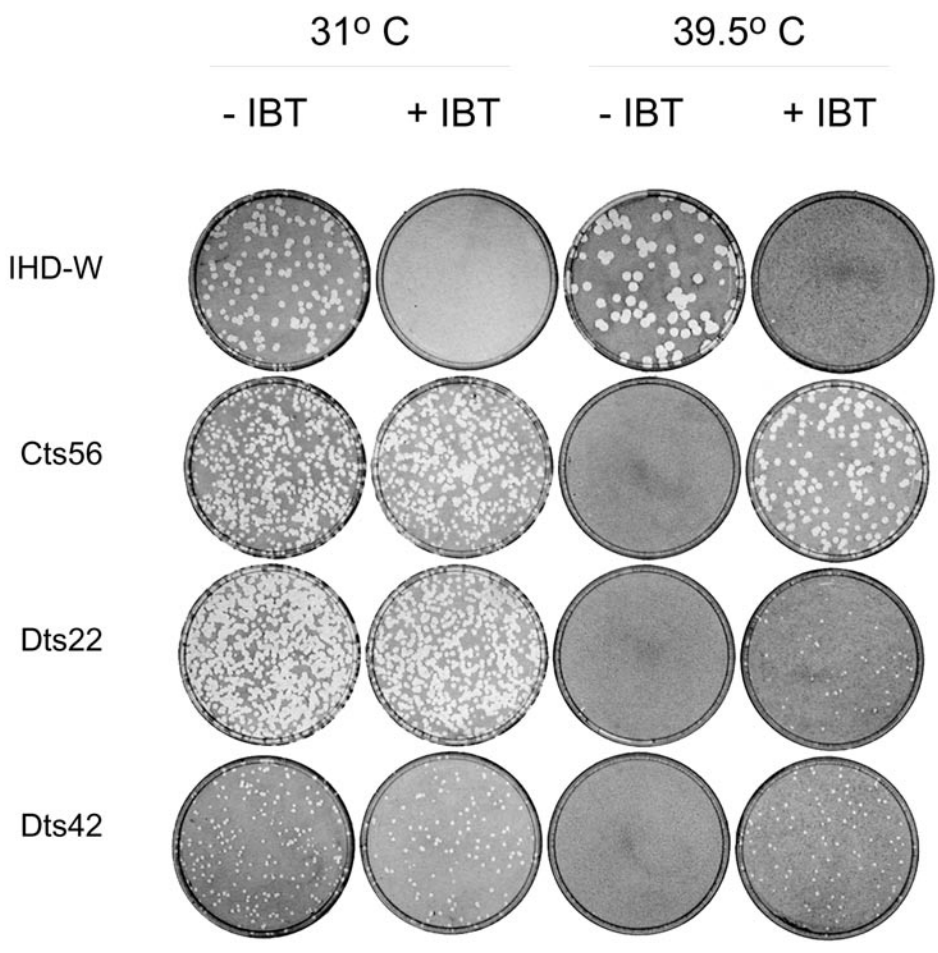

FIG. 2. Plaque formation of select G2 complementation group viruses. Plaque assays were incubated in the presence of 45 μM IBT (+IBT) or in the absence of drug (−IBT) at 31 and 39.5°C for 7 days and stained with crystal violet.

rescue of all six mutants using two cosmid clones and three plasmids covering the regions in question (data not shown). The cosmids pWR 67–98 and pWR 45–83 were shown previously to rescue Cts11 and localized the mutation within the region spanning genes *G6* to *G8* (Thompson and Condit, 1986; Meis and Condit, 1991). We obtained the plasmids pGEMA30WR, pGEMA30IHDJ, and pGEMA30TS that were used by Szajner *et al.* (2001) to map Dts46 to the *A30* gene. These plasmids contain the *A30* gene isolated from wt vaccinia WR, wt vaccinia IHD-J, and Dts46, respectively. Cts11, Dts68, and Dts89 rescued with pWR 45–83. In addition, Cts11 and Dts89 also rescued with pWR 67–98 (data not shown). These results are consistent with a map location, as previously described, in the G fragment. Rescue with Cts41 was ambiguous, resulting in no convincing mapping result. Dts45 and Dts46 both rescued with pGEMA30WR and pGEMA30IHDJ but not with pGEMA30TS, consistent with a map location in *A30* as previously described. To verify the marker rescue results, we sequenced the *A30* gene in all six viruses (data not shown). Sequencing revealed that the mutations in Dts45 and Dts46 were identical and located in amino acid 17, as previously reported for Dts46 (Szajner *et al.,* 2001). The *A30* gene from the remaining four mutants was wt. Although alternate explanations cannot be ruled out, the simplest interpretation of these results is that (1) three mutants, Cts11, Dts68, and Dts89, map to the right-hand side of *Hin*dIII fragment G as previously described for Cts11; (2) Dts45 and Dts46 map to A30, as previously described for Dts46; and (3) mutations in these two genes are noncomplementing. Aside from the fact that it does not contain an *A30* mutation, the map location of Cts41 is uncertain. We cannot rule out the possibility that all of these mutants are double mutants. In this case specifically, in addition to G fragment mutations, Cts11, Dts68, and Dts89 could potentially contain promoter mutations in *A30* that went undetected in our sequence analysis. Likewise, Dts45 and Dts46 could contain a second mutation in the *Hin*dIII G fragment. However, we consider it unlikely that this would have occurred in at least four individual mutant isolations; therefore, we favor the hypothesis that the G fragment mutants and *A30* mutants are noncomplementing.

The EM phenotype of Dts46 makes this mutant very unique. Dts46 was originally characterized by its overproduction of immature virus envelopes (Dales *et al.,*

1978). These envelopes are unique because they form separately from the other virus materials concentrated within the viroplasm and are assembled into multilayered spheres. The spicules that are normally associated with the adjacent bilayer membrane are disconnected in the Dts46 mutant. It was proposed that the spicules, designated p65E, were the product of the *D13L* gene. The unusual phenotype of this mutant provided evidence that under wt conditions the function of the spicules was to provide a rigid "exoskeleton" that determines the diameter and spherical form of the envelope surrounding immature virions (Essani *et al.,* 1982; Weinrich *et al.,* 1985). Cts11 and Cts41 were characterized as showing a normal phenotype (Condit and Motyczka, 1981; Condit *et al.,* 1983). This is not inconsistent with the EM phenotype described for Dts46; however, Cts11 and Cts41 have not been examined by electron microscopy.

I8: NPH-II

The members of the I8 complementation group include five mutants from the Condit collection (Cts10, Cts18, Cts38, Cts39, and Cts44) and one mutant from the Dales collection (Dts67) (Table 6). *I8R* encodes the NTP-dependent RNA helicase, NPH-II. Cts44 may contain another mutation outside of *I8R* because, upon marker rescue, the virus forms plaques that are consistently smaller than wt or the plaques of the other *I8R* rescued mutants. Sequencing revealed that Cts38 and Cts44 contain the same mutation in *I8R* (Fathi and Condit, 1991a; Gross and Shuman, 1996). Our complementation results indicated that Dts67 was noncomplementing with Cts38 but complemented Cts18 and Cts39. Thus, this is potentially another example of intragenic complementation as observed in gene *A24R* and discussed in more detail below.

I8R encodes the NTP-dependent RNA helicase NPH-II. NPH-II is one of five NTPases packages in virions, including NPH-I, VETF, A18, and the mRNA capping enzyme (Bayliss and Condit, 1995; Gross and Shuman, 1996; Yu *et al.,* 1997). The precise function of NPH-II is not known but it is essential for proper execution of early transcription by the core RNA polymerase. NPH-II may either disrupt the RNA–DNA hybrids that form during transcription or affect the extrusion of transcripts out of the viral core and into the cytoplasm for translation (Gross and Shuman, 1996).

The *I8R* gene is transcribed both early and late producing two 5′ coterminal transcripts. The longer transcript encodes the entire *I8R* gene, while the 3′ end of the smaller transcript maps to an early transcription termination signal located within the coding sequence. Only the large protein has been identified; the hypothetical small protein has never been observed. Three Condit mutants, Cts10, Cts18, and Cts39, contain mutations within the 5′ portion of the gene that would affect both the large and the (hypothetical) small proteins. Cts38 and Cts44 contain mutations in the 3′ region of the gene that would only affect the larger protein (Fathi and Condit, 1991a). There are formally two possibilities that could explain the apparent intragenic complementation within this group. First, it is possible that Cts38 is in fact a double mutant containing one mutation in *I8R* and a second mutation in another gene. In this case Dts67 might contain a mutation in the second (not *I8R*) gene affected in Cts38, thus accounting for its selective noncomplementation with Cts38. We favor the more interesting possibility that Dts67 is displaying intragenic complementation, implying that the *I8R* gene product acts as a multimer or is multifunctional. It is noteworthy in this regard that Cts38, which is the only mutant of the three Condit mutants that does not complement Dts67, is also the only mutant that would affect only the larger of the two potential I8 proteins.

Cts10, Cts18, Cts39, and Cts44 are all completely defective for growth at 40°C but have normal DNA and protein synthesis at both 31 and 40°C. Electron microscopy of Cts18 and Cts44 indicated that either mutant at 31°C is comparable to a wt infection at 31 or 40°C. At 40°C, all mutant-infected cells contained viroplasm with numerous clusters of apparently mature particles that resemble the mutant virions at 31°C. Thus there did not appear to be a defect in morphogenesis of Cts18 or Cts44 at the nonpermissive temperature (Fathi and Condit, 1991b). Particle-to-PFU ratios in Cts10 and Cts18 are higher than wt, indicating that a larger number of the progeny virions in a mutant infection at 40°C are noninfectious. In addition, the mutants Cts10, Cts18, and Cts39 packaged little or no NPH-II protein during assembly at 40°C (Gross and Shuman, 1996). Dts67 was characterized in both Dales groups H and Q of morphological aberrations (Table 1). Infection with Dts67 apparently resulted in some aberrations such as DNA paracrystals, aberrant membranes with and without spicules, and immature particles; however, immature and mature particles of normal appearance were still apparent, not inconsistent with the EM phenotypes of other I8 mutants.

J4: The 22-kDa RNAP subunit

The members of the J4 complementation group include two mutants from the Condit collection (Cts7, Cts20) and one mutant from the Dales collection (Dts44) (Table 6). The *J4R* gene encodes the 22-kDa subunit of the vaccinia RNA polymerase (Thompson *et al.,* 1989). Cts7 and Cts20, similar to mutants in other RNA polymerase subunits (A24 and J6), were characterized as defective late. At 40°C these mutants do not shut off early protein synthesis and are defective in late gene expression (Condit and Motyczka, 1981; Condit *et al.,* 1983).

The EM phenotype of Cts7 was examined at 31 and 40°C. At 31°C, the mutant infection was comparable to a wt infection. At 40°C, every cell contained at least one inclusion of viroplasm that was fairly uniform in density and lighter than the surrounding cytoplasm. These regions were devoid of cellular organelles and viral membranes or particles at any stage of maturation. Some membranes were present that did not appear to be viral membranes (Hooda-Dhingra *et al.,* 1989). The EM phenotype of RNA polymerase mutants, in general, shows the formation of large virosomes and no membrane structures and is consistent with a virus that is capable of replicating DNA but does not synthesize late viral proteins. The EM phenotype of Dts44 described by Dales is analogous to Cts7. Dts44 was characterized as group B mutants that produce foci of viroplasm at 40°C (Table 1) (Dales *et al.,* 1978). Thus, EM data are consistent with the complementation analysis. The phenotypes of the J4 mutants are similar to that of Cts53, a ts mutant in J6, the 147-kDa RNAP subunit.

J6: The 147-kDa subunit of the RNAP

The J6 mutants include three from the Condit collection (Cts51, Cts53, Cts65) and one from Dales collection (Dts85) (Table 6). *J6R* encodes the 147-kDa subunit of the viral RNA polymerase. Protein synthesis of each of the three Condit mutants demonstrated that Cts53 and Cts65 were strongly defective late at 40°C, and they synthesized little to no late transcripts at 40°C. Cts51, on the other hand, was "slightly" defective late in that the virus synthesized wt levels of late transcripts delayed by 2 to 4 h (Hooda-Dhingra *et al.,* 1989). This heterogeneity in phenotype is similar to mutants in *A24R*, the 132-kDa RNAP subunit.

Cells infected with Cts51 and Cts53 were examined by electron microscopy. At 31°C, both viruses possessed a phenotype comparable to wt. In Cts53 infections at 40°C, virtually every cell contained at least one inclusion of viroplasm, similar to the J4 mutant, Cts7. The inclusions contained some membranes that did not appear to be viral membranes. Cts51 infections at 40°C revealed viroplasmic inclusions that contained immature particles. These inclusions contained large, dense masses of viral nucleoprotein in close association with immature viral membranes. These structures are not evident in Cts53 infections, nor are they found in wt infections, consistent with a leakier phenotype that allows some late protein synthesis and morphogenesis (Hooda-Dhingra *et al.,* 1989). Dts85 was characterized by group C with the presence of rudimentary virus membranes containing spicules located in foci of viroplasm (Table 1) (Dales *et al.,* 1978). This phenotype is in agreement with the EM phenotype described for Cts51 and Cts53.

Unmapped complementation groups

Twenty-eight mutants comprising 21 complementation groups have not been mapped to a region or gene in the vaccinia virus genome. These mutants are identified in the map location column beginning with the letter U, indicating that the group remains unmapped, followed by an arbitrary number to help distinguish the groups from one another (Table 6). There is little information on most of these mutants other than their morphological phenotype. As demonstrated both by us and by other groups, the morphological phenotype does not necessarily provide clues to the genetic location of the mutation but is informative nonetheless.

Group U5 consists of four mutants from the Dales collection, Dts4, Dts8, Dts35, and Dts93. Complementation of the mutants in this group with Cts63 and Cts15 as positive controls is shown in Figs. 1b and 1d. The lack of complementation between Dts4, Dts8, Dts35, and Dts93 is demonstrated by the absence of plaques in row A, wells 5 and 6, row B, wells 1, 4, and 5, and row C well 2 (Figs. 1b and 1d). No description is available for Dts4 and no category of morphology was indicated for Dts93. The EM phenotype of a Dts8 infection describes immature particles with nucleoids and defective membranes with spicules (group L) (Table 1). A Dts35 infection is characterized by immature normal particles and mature particles with aberrant cores (group O) (Table 1) (Dales *et al.,* 1978).

Group U6 contains two mutants, only one of which was previously characterized. Dts16 was categorized as demonstrating immature normal particles and mature particles with cores that are predominantly transparent at the centers (group P) (Table 1). It was hypothesized that the transparent cores represented the absence of DNA and nucleoproteins (Dales *et al.,* 1978).

The two mutants representing group U8 were characterized by extreme opposite EM phenotypes (Table 1). Dts97 produced only foci of viroplasm (group B). Infection by Dts20 produced immature normal particles and mature particles with transparent cores (group P).

Group U10 is comprised of two previously uncharacterized mutants, Dts36 and Dts50. The phenotypes of these mutants have not been determined.

Dts77 is of particular interest for two reasons: (1) it is one of only six DNA negative mutants reported by Dales *et al.* (1980) and (2) there are discrepancies between our complementation results, the DNA negative phenotype as reported by Dales, and the mapping data reported by Millns *et al.* (1994). The DNA phenotype suggested that the defect in Dts77 was in a critical function required before the onset of DNA synthesis. Results obtained from the analysis of early viral DNA-binding proteins indicated that this mutant may be defective in functions involving early transcription or translation (Dales *et al.,* 1978; McFadden and Dales, 1980). The DNA-negative

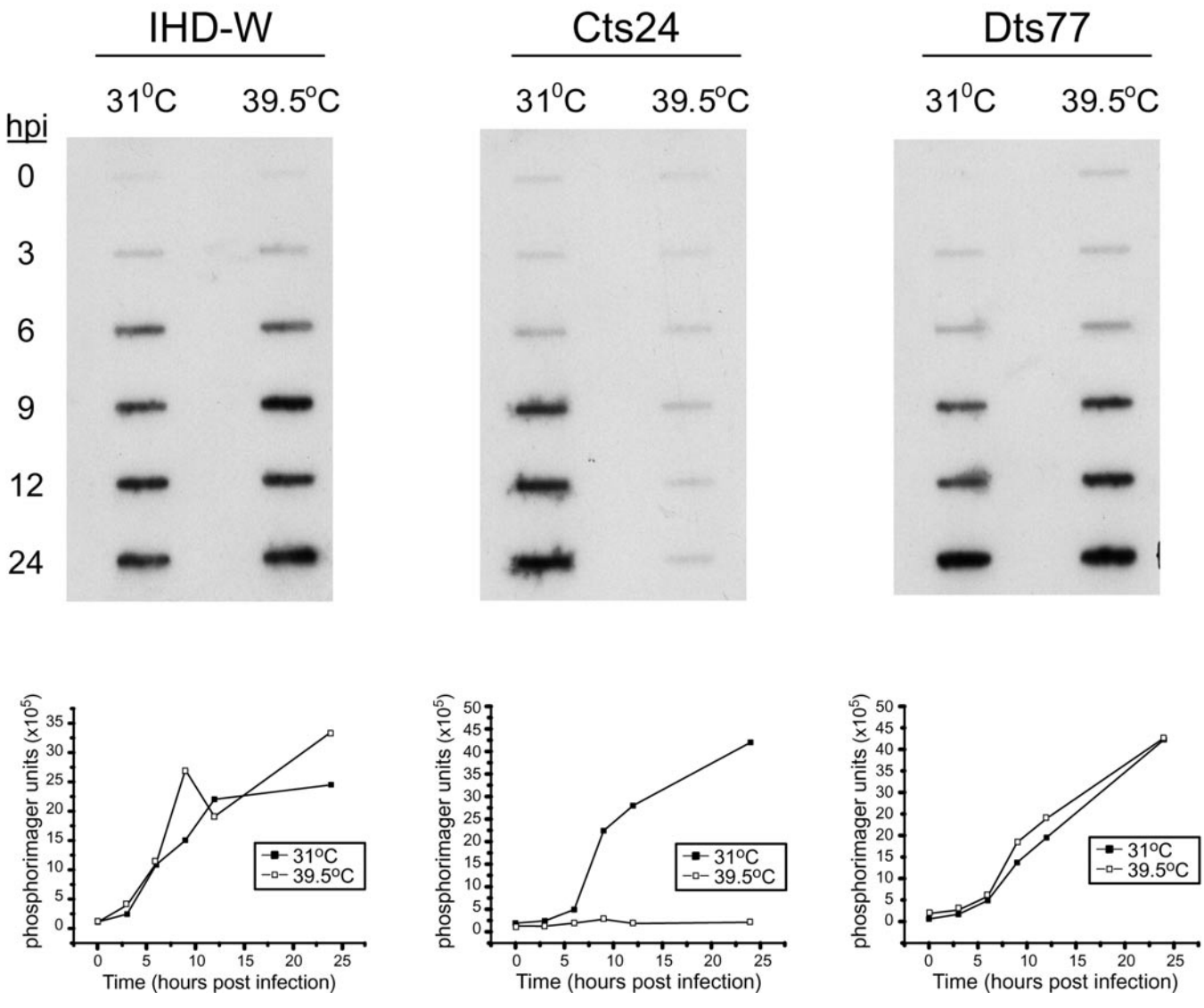

FIG. 3. DNA synthesis in IHD-W, Cts24, and Dts77 infections. BSC40 cell monolayers infected with wt IHD-W, Cts24, and Dts77 at 31 and 39.5°C harvested at 0, 3, 6, 9, 12, and 24 h postinfection (hpi). The samples were blotted onto a nylon membrane and probed with a virus-specific ^{32}P-labeled oligonucleotide mixture followed by autoradiography.

phenotype is inconsistent with mapping data that placed Dts77 in the *A3L* gene (Millns *et al.,* 1994). *A3L* encodes the precursor protein p4b that is expressed late during infection and processed to 4b, one of three major structural proteins in the virion core. (Millns *et al.* (1994) actually refer to the p4b gene as *A4L*; however, this is because of sequence differences between the Copenhagen and WR strains of vaccinia. We have adhered to the Copenhagen nomenclature throughout this article.) The two characterized mutants that map to *A3L*, Cts8 and Cts26, were described as having normal gene expression and DNA synthesis, an appropriate phenotype for a structural protein mutant (S. Kato, N. Moussatché, and R. C. Condit, unpublished observations). In addition, our complementation results do not support an assignment of Dts77 to A3 because, in our hands, Dts77 complemented Cts8, the representative from group A3. In an attempt to address these discrepancies, we analyzed the DNA synthesis by the Dts77 mutant in a time course from 0 to 24 h postinfection (hpi) (Fig. 3). DNA synthesis by Dts77 was compared to DNA synthesis by wt WR (not shown), wt IHD-W, and Cts24 (a DNA negative mutant in the *D5R* gene) at 31 and 39.5°C. Infected cell lysates from each time point (0, 3, 6, 9, 12, 24 hpi) were blotted onto a nylon membrane in a slot-blot apparatus and a ^{32}P-labeled oligonucleotide mixture was used for hybridization. In a wt infection (WR and IHD-W), DNA synthesis was first observed in the 6-h time point and continued through 24 hpi at both 31 and 39.5°C (Fig. 3, IHD-W). In a Cts24 infection, DNA synthesis is delayed at 31°C as compared to wt and absent at 39.5°C (Fig. 3, Cts24). In the Dts77 infection, DNA synthesis was first observed at

the 9-h time point at both 31 and 39.5°C and continued for the length of the time course. Therefore, although DNA synthesis in a Dts77 infection was slightly delayed, we classify it as a DNA-positive mutant. One possible explanation for the discrepancy between our results and those of Dales *et al.* (1978) and Millns *et al.* (1994) is that due to nomenclatural errors the groups may, in fact, be analyzing different viruses. Since we were unable to duplicate the complementation results reported by Millns *et al.* (1994), we chose to place Dts77 in the group U17 as an unmapped mutant.

Conclusions

The result of our complementation analysis is the identification of a renewed source of useful ts mutants in vaccinia virus. In addition to sorting 30 IHD-W ts mutants into 13 of Condit's existing complementation groups and confirming the previously described grouping of five Dales mutants in three complementation groups (groups A30, D4, and D12), we defined 18 new complementation groups containing 24 IHD-W ts mutant viruses. Given the abundance of research which has made use of the established Condit ts mutant collection, the collection described here, comprising 138 mutants in 53 complementation groups, enhances and expands this invaluable resource for further characterization of new genes that will lead to a further understanding of a vaccinia virus infection.

MATERIALS AND METHODS

Eukaryotic cells, viruses, and bacterial hosts

BSC40 cells, a continuous line of African green monkey kidney cells, and L_{929} cells (obtained from ATCC), a continuous line of murine fibroblast cells, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. The temperature-sensitive mutant viruses employed in this study were isolated and characterized previously (Dales *et al.,* 1978; Condit and Motyczka, 1981; Condit *et al.,* 1983). Wild-type WR and IHD-W (obtained from S. Dales in 1977) were initially grown and plaqued on both BSC40 and L_{929} cells at 31, 37, and 39.5°C. BSC40 cells were used for subsequent growth and titering of vaccinia virus WR and IHD-W ts mutants. For virological assays of the mutants, incubations were carried out at 31 and 39.5°C, the permissive and nonpermissive temperatures, respectively. Top10F′ *Escherichia coli* comp cells supplied in the AdvanTAge PCR Cloning Kit (Clontech Laboratories, Inc.) were used for cloning pTA-A24.

Plasmids and cosmids

pGEMA30WR, pGEMA30TS, and pGEMA30IHDJ were obtained from B. Moss (Szajner *et al.,* 2001). pTA-A24 contains the coding sequence for the vaccinia virus gene *A24R* inserted in the pT-Adv vector from the AdvanTAge PCR Cloning Kit (Clontech Laboratories, Inc.). The gene was PCR-amplified using gene-specific primers designed against the precise open reading frame of *A24R* from wt WR vaccinia virus DNA. Cosmid clones pWR 45–83, pWR 67–98, and pWR 112–149 were described previously (Thompson and Condit, 1986).

Complementation

Qualitative spot tests for complementation were performed as previously described (Condit and Motyczka, 1981). First, the appropriate amount of virus for use in complementation was determined by serial dilution in 24-well tissue culture dishes. Threefold dilutions from 10^{-2} to 2.43×10^{-4} for each mutant virus were tested by adding two drops of each dilution in a single well of a 24-well tissue culture dish containing confluent BSC40 cell monolayers. The dishes were then incubated at 39.5°C for 3 days and cell monolayers were stained with crystal violet. The highest concentration of virus that did not cause damage to the cell monolayer was chosen for use in complementation tests. Pairwise complementation assays were performed by the addition of one drop of the chosen dilution of each mutant virus to a single well of BSC40 cells growing in a 24-well dish. The dishes were incubated at 39.5°C for 3 days and stained with crystal violet. The dishes were then analyzed for the presence or absence of plaques.

Marker rescue

Marker rescue analysis was performed as a modification of the previously described protocol (Thompson and Condit, 1986). Briefly, 60-mm dishes of BSC40 cell monolayers were infected with 0.5 ml of the dilution of mutant virus used for complementation. Following a 1-h absorption at 31°C, the inoculum was removed and replaced with 4 ml 1× DME containing no serum. Infected monolayers were incubated at 31°C for 3 h at which time 0.1 ml Lipofectin (Invitrogen)-complexed DNA was added dropwise to the medium. The media was removed the next day and replaced with 4 ml 1× DME containing 10% fetal calf serum. The infected/transfected monolayers were then incubated at 39.5°C for an additional 3 days. On the fourth day of infection cell monolayers were stained with crystal violet and analyzed for the presence or absence of plaques.

Preparation of vaccinia virus DNA

Wt vaccinia WR DNA was purified from twenty 150-mm dishes of infected BSC40 cells as previously described with modifications (Esposito *et al.,* 1981). Infected cells were scraped from the dishes, collected by centrifugation, and resuspended in 40 ml isotonic buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA). The cells were collected by centrifugation at 2000 rpm, resus-

pended in 18 ml hypotonic buffer (10 mM Tris–HCl, pH 8.0, 10 mM KCl, 5 mM EDTA), and incubated on ice for 10 min. The plasma and outer viral membranes were dissolved with the addition of 50 μl β-mercaptoethanol and 2 ml 10% Triton X-100 and incubation on ice for 10 min. The cell debris was removed by centrifugation at 2000 rpm for 5 min. The supernatant was centrifuged for an additional 5 min and the supernatant was removed. Viral cores were pelleted from the remaining supernatant by centrifugation at 9000 rpm. The pellet was resuspended in 9 ml TE (10 mM Tris–HCl, 1 mM EDTA), 30 μl β-mercaptoethanol, 100 μl proteinase K, 400 μl 5 M NaCl, and 1 ml 10% SDS was added, and the solution was incubated for 60 min at 37°C. DNA was extracted twice with an equal volume of STE (0.1 M NaCl, 0.05 M Tris–HCl, pH 7.4, 0.001 M EDTA)-saturated phenol/chloroform followed by precipitation with 2.5 vol ethanol. The snarl of ethanol-precipitated DNA was picked from solution with the tip of a heat-sealed Pasteur pipet. The DNA was washed by swirling in a tube of 70% EtOH. The pellet was air-dried, resuspended in 0.2 ml TE (10 mM Tris–HCl, pH 7.4, 1 mM EDTA), and stored at 4°C.

Total cellular plus viral DNA from temperature-sensitive mutant viruses and wt IHD-W was prepared from 0.2 ml infected cell lysate using the QIAamp DNA mini kit (Qiagen) and resuspended in 200 μl Buffer AE (Qiagen).

DNA synthesis

Analysis of DNA synthesis was based on previously described protocols (Ausebel *et al.,* 1994; Cassetti *et al.,* 1998; Punjabi *et al.,* 2001). Monolayers of BSC40 cells in 35-mm dishes were infected with the appropriate virus at an m.o.i. of 10 and adsorbed for 30 min at either 31 or 39.5°C. The virus was then removed and replaced with 1× DME containing 10% fetal calf serum and returned to the appropriate temperature. The dishes of infected cells were incubated at either 31 or 39.5°C for 0, 3, 6, 9, 12, or 24 h postinfection. At each time point the cells were harvested, washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), suspended in 300 μl loading buffer (10× SSC, 1 M ammonium acetate (1× SSC is 0.15 M NaCl, 0.015 M Na-citrate)), and stored at −70°C until all samples were collected. Cells were lysed by three cycles of freeze-thaw and the samples were subsequently diluted with the addition of 450 μl loading buffer.

Samples were applied to a nylon membrane (Nytran N, Schleicher & Schuell) in a vacuum manifold (slot-blot apparatus, Schleicher & Schuell). The membrane was blotted three times on 3 MM Whatman paper saturated with 0.5 N NaOH, three times on paper saturated with 1 M Tris–Cl, pH 7.5–1.5 M NaCl, and finally three times on paper saturated with 2× SSC. The DNA was crosslinked to the membrane with a Stratalinker 2400 (Stratagene), sealed in a bag, and stored at −20°C until further use.

The probe used for DNA hybridization consisted of a mixture of 13 virus-specific oligonucleotides that were antisense to various regions of the *F17R*, *K1L*, *K2L*, and *A10L* genes (oligo mix). The probe was 5′-end labeled with [^{32}P]ATP using T_4 kinase (New England Biolabs). Unincorporated nucleotides were removed using a Nuctrap column (Stratagene).

Hybridization was carried out at 42°C using the protocol described by Ausebel *et al.* (1994) with slight modifications. The prehybridization and hybridization solutions contained 6× SSC, 0.1% SDS, 10× Denhardt's (50× Denhardt's is 1% (w/v) Ficoll 400, 1% (w/v) bovine serum albumin (Sigma, Fraction V), and 1% (w/v) polyvinylpyrrolidone), and 100 μg single-stranded Salmon sperm DNA. After hybridization, the membrane was washed in 5× SSC, 0.1% SDS once at room temperature and twice at 50°C for 20 min each, blotted dry, and exposed to X-ray film.

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